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THE LIFE CYCLE OF ASELOMARIS MICHAELI, A NEW GYMNOBLASTIC HYDROID

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The following account is of a hydroid not previously recorded, from the Atlantic coast of North America. It appears to be a form related to *Rhizorhagium* as redefined by Rees (1938), though not close enough to be included in that genus without a broadening of the definition and a reconsideration of the genera *Garveia* and *Bimeria*. Its closest relative appears to be *Atractylis arenosa* Alder (1862). According to Totton (1930), however, *Atractylis* is sunk in the synonymy of *Bougainvillia*, and *A. arenosa* is in any case in need of a new generic name. It is therefore proposed that the *Atractylis arenosa* of Alder become *Aselomaris arenosa*, and that the hydroid described here, which lacks the striking pseudohydrotheca and gelatinous perisarc of *A. arenosa*, be known as *Aselomaris michaeli*. This is in accordance with the views expressed by Rees and Totton (in personal correspondence). The genus *Aselomaris* is consequently defined as follows: bougainvillid hydroids with hydranths arisng singly from creeping stolons, with gonophores reduced to sporosacs and arising from the hydranth stalk, not from the stolons.

The present species was found throughout the general region of Boothbay Harbor, Maine, attached to the sides of floats, occasionally on Fucus fronds, in widely separated localities, namely, Lobster Cove, Townsend Gut, and the Town wharves. In every place it was associated with *Bougainvillia superciliaris* which it resembles in some respects, such as color, size and form of the hydranths.

Concerning its distribution, either it is an extremely local species or it has been extensively overlooked elsewhere. While a shallow water form, it is undoubtedly most inconspicuous. At the same time it should have been found if it occurred in the intensively collected Woods Hole region. Collections to the north have been more sporadic and its presence is therefore in doubt. It is quite possible that this is a northern species extending down to but not south of Cape Cod.

ASELOMARIS MICHAELI

This hydroid forms an encrusting mat often several centimetres square but with very little height. In fact most of the specimens obtained were collected only by shaving off the wood to which they were attached. The general nature of the hydranths is shown in Figure 1A. The hydranth grows vertically from the creeping stolon, the perisarc stopping short just above the base of the hydranth proper. It

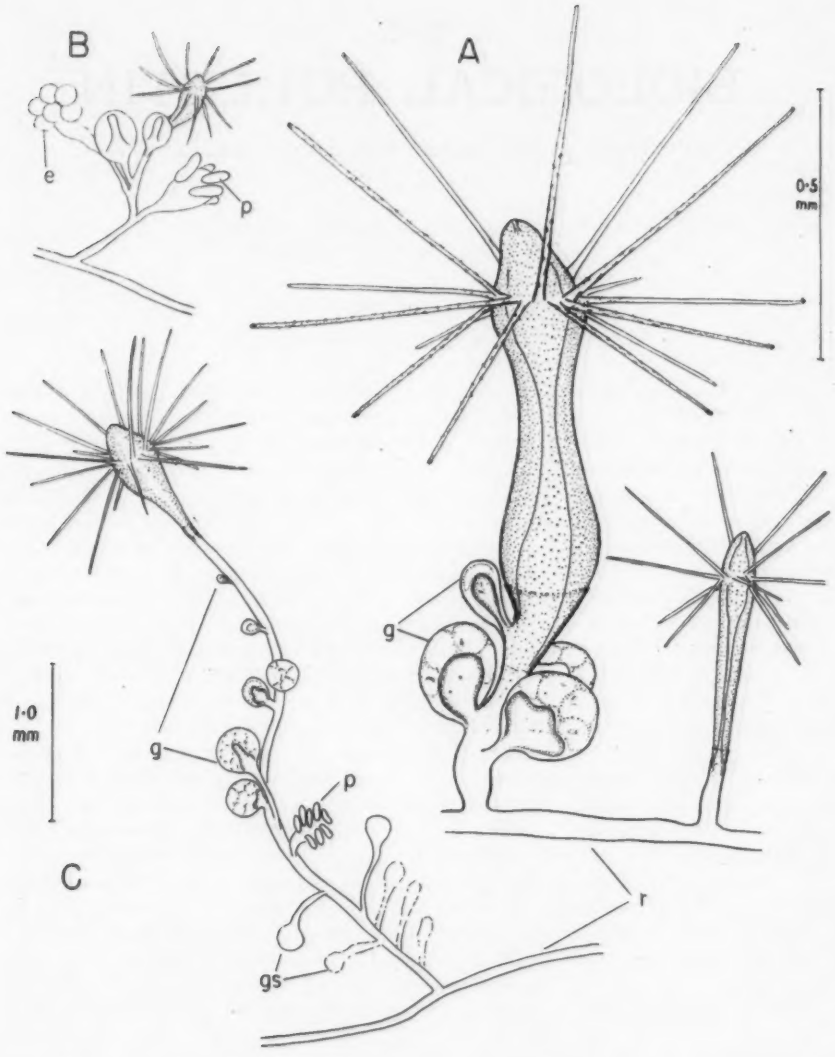

FIGURE 1. *Aselomaris michaeli.* A, large and small hydranths arising from the creeping hydrorhiza. B, somewhat older hydranth, at reduced scale, showing developing gonophores, gonophore with developing eggs and gonophore with planulae. C, maximum length hydranth, at same scale as B, showing succession of gonophores from distal juveniles to proximal stalk remnants. e, eggs; g, gonophore; gs, old gonophore stalk; p, planula; r, hydrorhiza.

has the form typical of the Bougainvillidae, with a conical manubrium and a single whorl of filiform tentacles. The tentacles are characteristically directed distally, laterally and proximally with virtually the same number in each category (Fig. 1A).

New hydranths grow only from the creeping stolon. Gonophore buds grow laterally from the base of the hydranth immediately proximal to the junction of naked and perisarc-covered coenosarc. This is a region of growth in a double sense. Not only are gonophores initiated, but the hydranth stalk grows in length, in effect pushing the growing gonophores down the stalk; that is, the new stalk that is progressively added proximally secretes chitinous perisarc and carries with it the growing gonophore, while new material and new gonophores are successively added between the first formed gonophore and the hydranth base. Thus we get a continually lengthening stalk bearing a series of gonophores, youngest nearest the base of the hydranth and oldest near the junction of the stalk and hydrorhiza (Fig. 1C).

No branches are given off and the hydrorhiza forms a closely applied creeping stolon. The only outgrowths are the buds arising from the hydrorhiza that develop directly into hydranths; that is, gonophores do not develop directly from the hydrorhiza, but only single hydranths; whereas hydranths only rarely form as lateral branches of the stalk, in place of a gonophore.

In an old hydranth as many as fifteen gonophores may be present, the youngest near the hydranth base being in early stages of devolpment, those in the middle zone being close to or actually functional, and those near the base of the stalk being represented mainly by stumps within a persisting perisarc.

Female Gonophores

Gonophores first appear at the base of the hydranth at the region where the hypertrophied gastrodermis of the hydranth thins down to that characteristic of the coenosarc. They arise one at a time as a two-layered protrusion of the body wall. The entocodon, which is apparently of epidermal origin, arises relatively early. By the time the next in series is starting to form, a given gonophore has its entocodon differentiated almost entirely into about eight or ten oocytes. The more opaque endodermal component becomes somewhat pointed at the center, foreshadowing the spadix (Fig. 2B). With further growth the spadix forms a high cone, reaching to the distal epidermis of the gonophore (Fig. 2C). At its maximum size (Fig. 2D), the oocytes are full grown with the germinal vesicle of each lying in the part farthest away from the spadix. The gonophore stalk is greatly lengthened and is about twice as long as the diameter of the gonophore proper. Rhythmical movements or a writhing of the gonophore commences at this stage, at least under the conditions of microscopic examination. The contained eggs change shape with the movements and may appear themselves to be responsible for them, but a close examination reveals the activity to reside in the layer of the gonophore wall immediately beneath the epidermis and derived from the entocodon. In mature gonophores the writhing movements and contractions of the wall culminate in its rupture distally (Fig. 2E). This rupture has two consequences. Distally, where the cells become greatly stretched and thinned out, rupture results mainly in their disaggregation, so that instead of withdrawing as a sheet they remain as isolated spherical cells scattered over the sticky surface of the ripe eggs. The proximal part of the wall

however does withdraw towards the base of the spadix, leaving the latter naked and still holding on to the eggs by adhesion. At the same time the epidermis, with its tension now released, contracts down the stalk, and where the stalk narrows the epidermis becomes somewhat thickened and wrinkled (Fig. 2F, G). The eggs are fertilized and undergo their cleavage and development up to the planula stage ad-

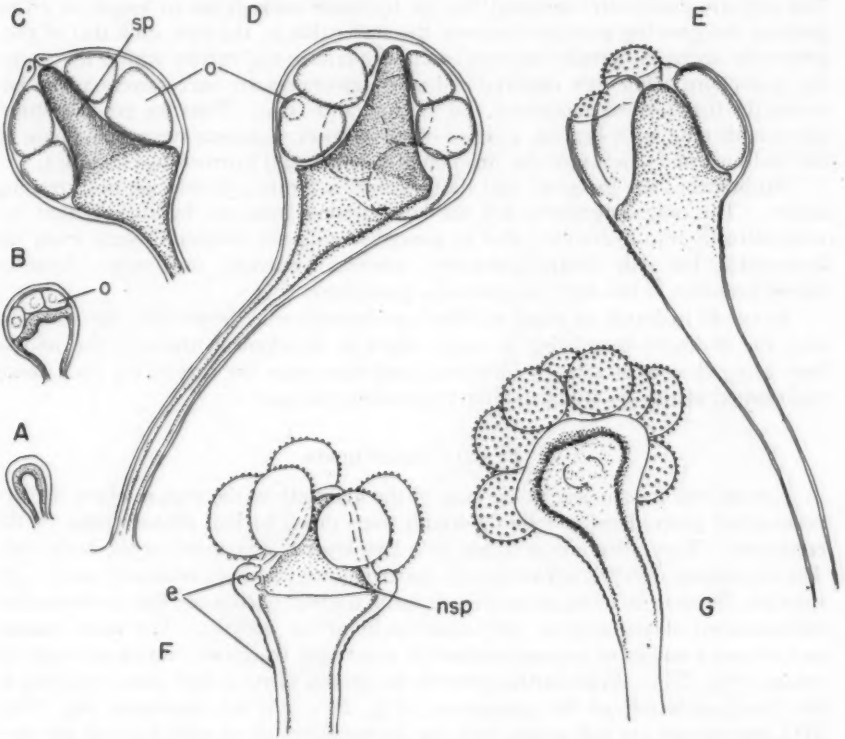

FIGURE 2. Female gonophores. A, young gonophore before appearance of entocodon. B, after segregation of ova from entocodon. C, D, fully formed gonophores with large ova and well developed spadix and long stalk. E, distal disintegration and rupture of epidermis, and basal epidermal contraction. F, G, matured gonophores with fully retracted epidermis, bearing fertilized eggs attached to the denuded spadix. e, contracted epidermis; nsp, naked spadix; o, ova; sp, spadix.

hering to the gonophore spadix, remaining there until able to move away as a result of their own efforts. Planulae on the point of departure are shown in Figure 1B, C and Figure 4A. Throughout their development on the spadix, an internal active hydroplasmic streaming is maintained within the lumen of the spadix. Whether of course this streaming is of any value to the developing eggs is difficult to determine.

Male Gonophores

The male gonophores develop as do the female, and have essentially the same appearance. Early stages showing the entocodon and the formation of the spadix are shown in Figure 3A and B. The lumen of the spadix alternately expands and

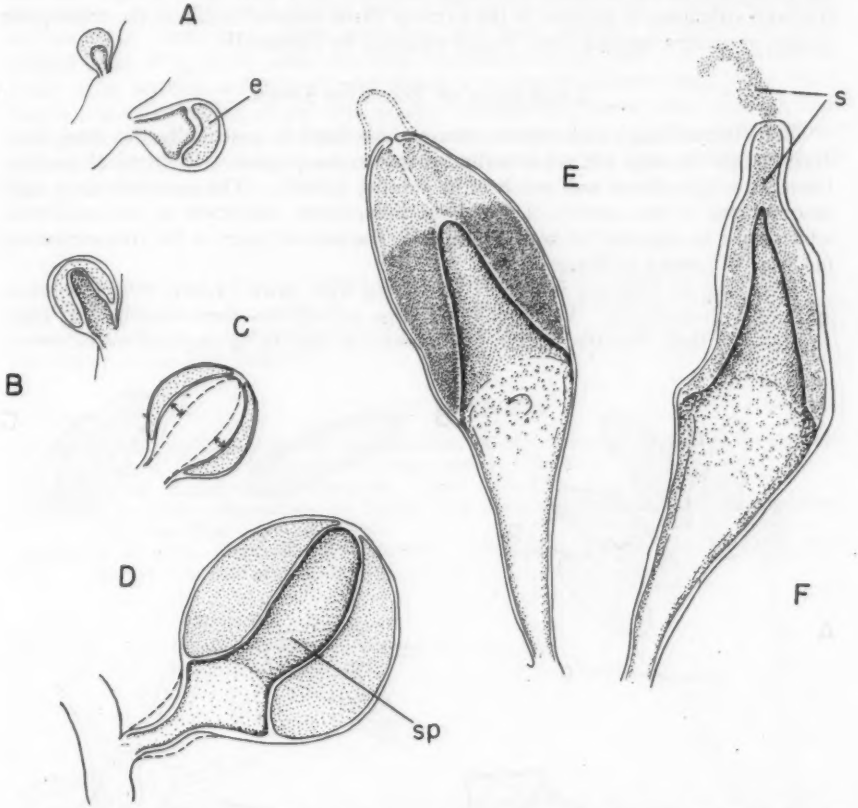

Figure 3. Male gonophores. A, B, young stage showing formation of entocodon and germ mass. C, later stage with arrows indicating expansion-contraction amplitude of spadix. D, late stage with germ cells present as spermatids, and broken lines indicating stalk diameter when dilated. E, ripe gonophore with active spermatozoa and expanding and contracting distal end. F, emission of spermatozoa. e, entocodon; sp, spadix.

contracts in a regular manner, with an amplitude indicated by the arrows in Figure 3C. In the later stages (Fig. 3D) the dilatation and contraction is more obvious in the short stalk of the gonophore, the growing mass of male germ cells possibly inhibiting or at least reducing the freedom of movement of the spadix wall itself.

With the attainment of full size, not only is the stalk relatively long, but the distal part of the gonophore also elongated. This is due on the one hand to the contractile property of the wall of the mature gonophore, and on the other to the pseudo-fluid quality of the mass of ripe germ cells. There is a rhythmical contraction of the gonophore wall similar to that of the female gonophore, but here resulting in an alternation between the stages indicated in Figure 3E. Finally the contractions culminate in rupture at the extreme distal pointed end, and the consequent escape of mature spermatozoa, shown escaping in Figure 3F.

Liberation of Eggs and Sperm

Both mature eggs and spermatozoa are liberated in essentially the same way, even though the eggs are not actually set free in the process. Rhythmical contractions of the gonophore wall result in its rupture distally. The contractions in each case are due to the activity of the tissue immediately subjacent to the epidermis, which must be regarded as homologous with the muscle layer of the free-swimming medusae of species of Bougainvillia.

Spawning in hydroids is usually associated with dawn (Lowe, 1926) or dusk, and not with darkness. Yet most of the ripe gonophores were examined at night and most of them ruptured within five minutes of first being exposed to the micro-

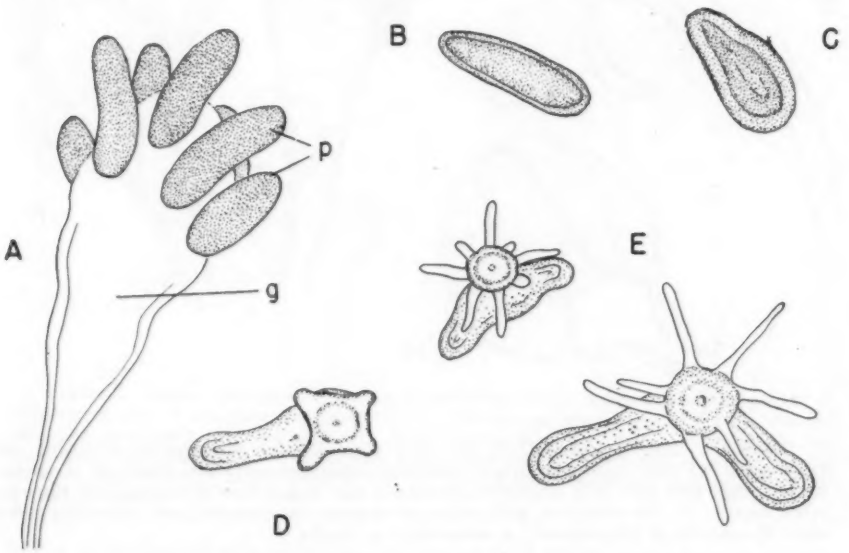

Figure 4. Development of planula. A, planulae about to swim away from gonophore. B, free-swimming planula. C, planula after about 24 hours, changing shape and losing ciliation. D, 12 hours after attachment, with stolon and 4-tentacled hydranth. E, two individuals 24 hours after attachment, with bipolar stolon on hydrorhiza and 8-tentacled hydranth. g, gonophore; p, planula.

scope light. It appears probable therefore that the stimulus of light evokes the contractions of the muscle layer, and spawning inevitably follows.

Settling of the Planula

Planulae escape from their adhesion to the spadix only as they become ciliated and active. A set about to launch forth is shown in Figure 4A. A planula swims for about 24 hours as a ciliate organism, and then becomes progressively pear-shaped (Fig. 4C), at the same time resorbing the external coat of cilia. About 12 hours after settling, a hydranth and stolon are already differentiated (Fig. 4D), four tentacles emerging in the first place. Twenty-four hours after settling, four intermediate tentacles are usually well formed, or a total of eight, while stolonic growth is bipolar, growing in opposite directions from the base of the hydranth along the substratum (Fig. 4E).

Summary

A new species of a hydroid genus not previously recorded from the Atlantic coast, *Aselomaris michaeli,* is described. The development and activity of both male and female gonophores are described in detail, together with settling of the planula and formation of the first hydranth.

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NOTE ON THE SPAWNING OF THE HOLOTHURIAN, THYONE BRIAREUS (LESUEUR)

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Thyone briareus is fairly abundant at Woods Hole, Massachusetts, yet very little has been recorded about its spawning. The present note is based upon observations made in the laboratory in 1948.

The Breeding Season

June is the principal month in which shedding has been observed in the laboratory at Woods Hole. Pearse (1909) noted spawning from June 22nd to July 5th, Ohshima (1925) reported it from June 21st to 24th in 1921, and Just (1929) observed it repeatedly during the month of June for several years. Mead (1898) found every animal full of nearly ripe eggs or sperm on April 24th, which would suggest a season beginning earlier than June, but Just (1929) claimed that eggs obtained in April and May were unripe ovocytes, capable of responding to insemination but unable to develop. There are no other data for the early part of the breeding season. As to the latter part, Pearse's observation of shedding on July 5th is the latest specific data published for *Thyone* at Woods Hole, although Bumpus (1898) remarked that the breeding season was probably June and July and Clark (1902) stated that *Thyone* apparently bred in the summer.

A study of 314 animals was made during the second half of June, 1948. Forty-nine of these animals shed in the laboratory, and 215 which did not shed were dissected. The results are summarized in Table I. The table shows the number of animals collected on each date and their condition as determined by shedding or dissection, together with estimates of shedding capacity based on the findings in these two categories. Line 8 in the horizontal direction includes some cases of undetermined sex among spent animals. When spawning is over, the gonadal tubules are so small that a careful microscopical examination is necessary to determine the sex. Such examinations were not made in the cases indicated. The single case of undetermined sex among the partly spent animals of the June 19th group was simply the result of an oversight. It is quite easy to distinguish the sexes when partly spent animals are dissected. The gonadal tubules of the male are an opaque yellowish or orange color and are pointed at the distal end, while those of the female are more translucent, golden or mustard colored, and have blunt distal ends. The dates of collection shown in the table for the different groups of animals do not necessarily indicate the dates on which spawning occurred. In fact, owing partly to experimental conditions, sheddings usually took place on subsequent dates. Nevertheless, the sheddings are listed as of the date of collection inasmuch as they indicate, no matter when they occurred, the shedding *capacity* of animals collected at that time. For example, some animals collected on June 15th were

TABLE I *

Shedding data and estimates of shedding capacity. (Explanation of categories in text.) Since shedding occurred under various experimental conditions the numbers in line 5 do not necessarily reflect maximum shedding as it might have occurred in nature.

1. Group	1		2			3			4			5			6			8			10		
2. Date	June 15		June 19			June 21 or earlier			June 21 or earlier			June 23			June 26			June 29			July 15		
3. Number in group	24		64			35			48			36			63			20			24		
4. Sex	♀	?	♂	♀	?	♂	♀	?	♂	♀	?	♂	♀	?	♂	♀	?	♂	♀	?	♂	♀	?	♂
5. Number shed	1		8	3		3	3		15	5		5	0		0	2		3	0		1	0		0
Number dissected																								
6. Full	0		0	5		0	0		0	2		0	1		0	0		0	1		0	0		0
7. Partly spent	4		5	7	1	14	1		2	0		7	0		4	7		13	0		4	0		3
8. Spent	1		0	11	4	15	2		1	7		6	15		9	21		11	6		4	10	6	5
9. Remainder estimated sheddable	1.3		3.4	.23		.26	1.8		7.8	3.8		6.0	.24		.96	.93		1.66	.25		1.25	0		0
10. Estimated spent	.3			.5			1.4			6.2			5.8			3.4			2.5			0	
11. Number of each sex estimated in group	10.9		13.1	29.1		34.9	15.9		19.1	21.8		26.2	16.3		19.6	28.6		34.3	9.1		10.9	10.9		13.1
12. Number of each sex estimated sheddable	6.3		16.4	15.2		17.3	5.8		24.8	10.8		15.5	1.3		4.96	9.9		17.7	1.3		6.3	0		3
13. Per cent of each sex estimated sheddable	57.9		125.5	52.4		49.5	36.7		129.9	49.4		59.3	7.6		25.3	34.6		51.5	13.8		57.3	0		22.9

* Note. Total number of females (shed and dissected) = 115, or 45.4 per cent females
Total number of males (shed and dissected) = 138, or 54.5 per cent males
63.5 per cent of the females studied were spent
36.9 per cent of the males studied were spent
7.8 per cent of the females studied were full of eggs
None of the males studied were full of sperm

found shedding on the 18th, others not until the 22nd, 23rd and even the 26th of June, yet all of these sheddings indicate the shedding capacity of animals collected on the 15th. Compared with specimens actually collected on the 23rd or 26th, the June 15th group shows a much higher shedding capacity. The numbers collected were meager for some of the dates studied but an analysis of the table does show, in a general way at least, the probability of obtaining embryological material at this season. It may be mentioned that no single season is necessarily typical, since there is considerable variation in weather conditions during the spring months from year to year and this is probably reflected in the environment of the *Thyone* beds. A comparison of water temperatures at Woods Hole for the years 1902 through 1906 is given by Sumner, Osburn, and Cole (1911) and the air and water temperatures of recent years are on file at the Marine Biological Laboratory and the Woods Hole Oceanographic Institute (unpublished).

It has not been found possible to determine the sex of *Thyone briareus* by external inspection. Certainly there are no obvious correlations between sex and size, color, general appearance, or behavior. Therefore it is assumed that the specimens selected for dissection represented a random sampling of the entire group and showed about the same proportions of the two sexes as would any other sampling, as, for instance, the specimens left undissected. From lines 5, 6, 7 and 8 it can be seen that a total of 115 females were found, through shedding and dissection, while there were 138 males. Obviously 253 animals are not enough for a very accurate study of the sex ratio but, for want of more, this number must be used at present. Hence, it is concluded that 45.4 per cent of the animals would be females and 54.5 per cent would be males, or a ratio of about five to six, in any group of *Thyone* collected as were the subjects of this study.

Estimates of shedding capacity within a group were obtained in the following manner. (1) Animals which actually did shed were considered sheddable as of the date collected. (2) Some animals were probably prevented from shedding by the conditions to which they were subjected. If dissection showed the gonads full of seemingly mature gametes, the animals were considered sheddable. This seemed permissible since shedding did occur in the first group collected. (3) It was found subsequently that a given animal can shed more than once, at intervals of several days. Therefore, dissected animals found to be only partly spent were also considered sheddable. (4) Some animals that did not shed were not dissected, but an estimate was made showing those that should have been able to shed. (The number not examined was multiplied by the proportion found sheddable among those that were examined.) The sum of these four categories gave the estimated number of animals able to shed, from which the percentage of shedding capacity for the group could be calculated. However, since there were more males than females and since, moreover, most groups showed more females than males to be already spent, the shedding capacity was estimated in terms of percent of each sex capable of shedding. This seemed worth while in spite of the inaccuracies bound to arise from a study of such small numbers (e.g. Group 3 shows 129.9 per cent sheddable males), because the low female shedding capacity would otherwise have been masked by the higher male activity.

Table I shows that nearly half of the females examined in the middle of June had already shed their eggs. By June 21st more than half were spent, and as early

as June 23rd one group showed the exceptionally low shedding capacity of only 7.6 per cent. In the last week of June, little more than a third of the females could shed and much lower percentages might be expected, such as the 13.8 per cent noted on June 29th. By July 15th there were no females able to shed, out of a group of 24 animals examined, although three of the males could still have shed some sperm. Aside from the numerical evidence of a waning season, the quantities of mature germ cells, either shed or found by dissection, dwindled as the month progressed. It seems, then, that the guess of Bumpus about shedding in June and July in 1898 would only have proved half accurate in 1948. And while one might have expected an occasional shedding of eggs as late as July 5th, as Pearse reported in 1909, that date could not have been recommended for the purpose this year. In fact, it would not have been worth while to attempt a detailed examination of the eggs of *Thyone* from animals collected after the third week of June.

Although this study was not begun until the middle of June, there are some indications of what may have occurred earlier. One might expect a higher percentage of mature females to be capable of shedding at the height of the season than actually did shed at any time from mid-June on. Nearly half of the batch was spent as early as June 19th. As early as June 15th there were as many partially spent as there were shedding, and on the 19th and again on the 23rd, there were more partially spent than the sum of the shedding and fully ripe ones combined. Certainly the season was waning during the entire second half of June. All this suggests a peak reached before June 15th. If this is correct, then the spawning season must either have been very short or have begun before June 1st. We have Mead's (1898) observation to support the latter possibility and Just's (1929) to support the former. Until the question is settled, the most promising time for successful embryological collecting must be considered to be the first half of June or possibly the middle two weeks of this month.

Shedding

Before it begins to shed, an animal expands greatly, to perhaps twice its former length, and starts waving its tentacles gently in the water in "feeding movements," as noted by Pearse in 1909. The position of the body can vary from vertical to horizontal, attached to the bottom or the sides of its container. It needs enough free space for the extended tentacles because sudden contact with any object will usually cause contraction, which might possibly delay shedding. On the other hand, shedding is not impossible in cramped quarters and once begun, under whatever circumstances, the process can be resumed in spite of various interruptions, such as removing the animal from water, leaving it practically dry, placing it under a very bright light, rinsing it in cold tap water, and so on. However, the writhing movements of an animal out of water or in too small a container will disperse the germ cells and could injure them.

As has been mentioned above, the sexes are indistinguishable externally. In both, the genital pore lies at the tip of an inconspicuous little stalk in the mid-dorsal line, between the bases of two large tentacles. It is easily located since it occupies the point on the tentacular ring diametrically opposite the only pair of small tentacles. The genital duct connects the pore with the gonads, which lie along the dorsal body wall, about halfway between the mouth and anus. Kille (1939) has de-

scribed the gonads in post-spawning animals and discusses the tubules and their contents as found in July and August. It seems as if the entire gonadal knot need not be involved with spawning each year and some tubules may remain small and undistended. On the other hand this may be simply the appearance of late-season tubules. Ohshima (1925) described irregular masses of disintegrating yolk in the ovarian tubes, concluding that they were probably eggs which had failed to be laid in the previous spawning season and were undergoing degeneration. Brownish masses were especially notable in the ovarian tubules of spent females toward the end of the present study and these too were given Ohshima's interpretation, but it was thought that they probably represented degenerating eggs of the current season. Kille suggested that some large tubules may have been lost by some of his animals seen in July and August, an impression also gained from the present study, but not actually proved.

Once when an animal was vastly expanded and undergoing the writhing movements that precede shedding, the body wall became semi-transparent and the genital duct could be seen, a straight, grayish line leading to the pore. It showed clearly even when the animal was removed from the water and held up to the light. Shedding finally did occur and the animal proved to be a female as had been predicted. After the shedding the line was gone, but later a shorter line was noticed in the same region. Since the eggs are grayish and the sperm white, it is possible that some method of sex diagnosis might be worked out, but it probably would be practical only with full, mature animals. It was not effective this season.

The germ cells are emitted in a slow, fountain-like stream, so that they rise upward for a very short distance and then fall toward the bottom of the container. Since the tentacles are waving, there is fairly rapid dispersal, especially of the sperm. Moreover, animals nearby in the same container will ingest the gametes being shed by a neighbor. A female which has been in a container with a shedding male can be partially freed of sperm by rinsing in running tap water; but since the tentacles are withdrawn during this process, all the sperm cannot be reached and some of her eggs, if shed soon, are certain to be fertilized. Many changes of sea water with intervals for feeding movements in each would cut down the possibility of sperm contamination, but not eliminate it. Probably the best way to obtain unfertilized eggs under these circumstances is to hold a slender medicine dropper directly above the genital papilla. If introduced slowly and gently this is quite feasible, although tedious. It is much easier to keep prospective shedders in separate containers. Segregation is desirable even if fertilized eggs are to be collected, since the relatively large amount of sperm, even in 6 by 9 inch battery jars, will literally smother the eggs and is to be avoided.

The duration of the shedding process varied considerably, perhaps depending upon the age, and hence size, of an animal as well as on its degree of fullness. When watched, the process was sometimes as short as 10 or 15 minutes but extended up to four and a half hours in the largest shedding witnessed. Usually it was completed within about a half hour of the time of starting. Sometimes shedding is not quite continuous even when apparently undisturbed, and will proceed intermittently with intervals of from a few minutes on up to nearly three days. These latter repeated sheddings were noted several times, in one unusual case 7 and 9 days, re-

spectively, after the animal was collected. Repeated shedding has not yet been found in a female.

The present study confirms the statements of Ohshima (1925) and Just (1929) that eggs obtained by means other than natural shedding connot be fertilized in the laboratory. It is easy to secure large eggs simply by mincing the ovarian tubules. The eggs which fall out are about as large as shed eggs. They contain a large germinal vesicle which has never been seen to break down except under pressure, applied externally. They have not been fertilized.

Factors in Shedding

No reliable method has been found to induce shedding at the will of the investigator, but various factors which might influence the phenomenon have been examined in connection with the shedding of the 35 males and 14 females which took place during the present study. The preponderance of males shedding was much greater than might be expected on the basis of the sex ratio. Perhaps they simply shed more easily or more frequently. Perhaps their activity lasts longer during the declining season. Whatever the reason, it should be borne in mind that the factors considered were effective on males particularly. They cannot be clearly demonstrated to be shedding agents until they have been examined again, at a time when many ripe females are available.

(*1*) *Time of Day*

Ohshima (1925) found that shedding always occurred late in the afternoon of the day the animals were brought into the laboratory. Once in 1948 a number of animals did shed around 6:00 P.M. of the day they were delivered, but it happened that this batch had been collected previously and kept on the water table in the supply department for at least a day. By far the majority of sheddings watched this year took place in the evening, mostly around 8:00 and 9:00 o'clock, and some as late as 1:00 or 2:00 A.M. Of course, many of these sheddings occurred under experimental conditions and could not be attributed entirely to a natural tendency. It did seem, however, as if there might have been a rather strong natural bent toward evening shedding which persisted despite external conditions. Nevertheless, at least two animals were seen shedding in the morning, one between 9:00 and 10:00 o'clock and the other at 12:00. Therefore, this part of the day need not be ruled out as a possible time for obtaining the germ cells.

(*2*) *Light*

Ohshima was able to induce animals to shed during the day by placing them in a dim light. Returned to bright light, they ceased shedding but would continue again if replaced in subdued light. Utter darkness did not cause shedding. Presumably these observations were made during the four days when Ohshima obtained eggs. During the present study sheddings were observed on at least ten different days and under many circumstances. They were seen to take place in natural, indirect daylight, in subdued daylight and in electric light of various intensities from rather subdued to bright lamplight directed right on the animals. They were also found to have occurred in complete darkness. Several times a

male which had started to shed was picked up unceremoniously and transferred to a new dish under a bright electric lamp. Soon it started to shed again. Some females shed in bright light and their performance was watched in light sufficiently bright to allow one to see it easily, but it is true that at least 8 of the 14 females studied did shed in either complete or semi-darkness. The number is too small to be significant. This diversity of findings seems to rule out subdued light as a prerequisite. Possibly Ohshima's observations can be explained in the following way.

Thyone is very sensitive to changes in light which occur suddenly, as when, as Pearse showed (1908), a shadow passes abruptly between the animal and the light source. It also reacts immediately to jarring. The reaction to both stimuli is the same: contraction and in-drawing of tentacles, enough to interrupt shedding. It may take a minute or longer before the animal will expand again after such a contraction. If the shedding time were a short one, interruptions such as these would be enough to appear to stop the process. With only four days of observations, and eggs to collect at the same time, Ohshima may have returned his specimens to the dim light before they had had time to start shedding in the other types of light investigated. On the dates of his collections, June 21st to 24th, he might also have been dealing with some animals that were partly spent and had little to shed, as in the present study.

(*3*) *Presence of a Male*

Finally, Ohshima found no females spawning spontaneously, but only after the emission of sperm by nearby males in the same container. This year it was inconvenient to isolate all the animals of a group because of the container requirements for animals of this size, but at least two females did start to shed while wholly isolated in separate bowls. Others, isolated as soon as shedding was noticed, would continue after isolation, and this was, incidentally, usually after a rinsing in cold tap water too. The male effect was tested conversely when a female which had shed two days earlier was placed in a jar of freshly shed sperm, in an attempt to induce further shedding. It did not occur, even though subsequent dissection showed at least some mature eggs in at least some ovarian tubes. It would be more conclusive to use a full, mature female, but as yet no way has been found to distinguish such animals. At all events, isolated females are perfectly capable of initiating shedding.

(*4*) *Size*

The size of these animals is very hard to determine since so much depends upon their water content at the time of examination. However, any animals known to the supply department as medium or large will probably prove satisfactory as a source of germ cells, in season. It is not always the largest in a batch that will shed first, nor is the size any criterion of the sex of an animal. Very small specimens are to be eschewed for several reasons. (a) If mature, they will have only a small quantity of germ cells. (b) They may be mature animals already spent and hence reduced in bulk by the absence of distended gonads. (c) They may be small because they have eviscerated and hence are lacking in most of their viscera, usually including the larger gonad tubules. All three types have been encountered in the present study, (a) and (b) most frequently.

(5) Quantity of Sea Water

Just (1929) observed spawning repeatedly among animals if kept in large quantities of sea water. This year, shedding would begin in any amount of sea water sufficient to hold the animal and allow it to expand, and once begun, several males were found able to continue shedding even when placed in practically dry finger bowls in the hope of stopping them temporarily. They provided a little water for themselves, from the cloaca. Sometimes an animal barely submerged in a four-inch, shallow bowl would undergo the expansion that usually precedes shedding and would virtually double up on itself in the cramped space. When noted, this was remedied out of sympathy and the animals went on to shed in larger quarters, but sometimes they were not moved, and shed in the small containers. Usually, however, shedding was observed among animals in the 6 by 9 inch or 6 by 7½ inch cylindrical battery jars which proved to be the most suitable containers for them. Here there was room for maximum expansion but at the same time some hope of retrieving the shed cells.

The water was usually changed several times a day to ensure freshness but it is questionable whether this is really necessary if the animals are stored in a cool place (15°–17° C.). One sturdy specimen was found shedding after at least two days in unchanged sea water at about 20° C. Pearse (1908) and others have shown that *Thyone* can endure a good deal of variation in the environment, such as higher salt concentration, dilution with fresh water, excessive heat (some survived more than two hours' exposure to as high as 37° C.), or exposure to air. It is unlikely, then, that large quantities of sea water are essential to shedding. However, it is probably desirable for storage, since animals stored in running sea water did shed, even many days after collection. It would be very hard to gather eggs or sperm shed at random in a large aquarium and, as a matter of fact, no sheddings were observed among stored animals. Possibly they escaped notice because of rapid dissipation of the gametes.

(6) Freshness of Animals

It is generally held that fresh, well-fed animals provide the best embryological material. In the case of *Thyone* there is one drawback to using animals just collected. Their habit of pumping mud through the gut soon clouds the water so that gametes would be very hard to see, if present. The waving of the tentacles provides an excellent stirring system which keeps the debris from settling. An animal which has been stored in a large aquarium, or in running sea water, overnight or longer, has got rid of much of this material and is an easier one in which to watch shedding. Spawning often does occur several days after collection and in a few cases animals which had been in the laboratory as long as nine and ten days were found shedding. Normal larvae developed from eggs shed two or three days after the parents were collected. The problem, then, is to keep the animals from shedding until after they have emptied their digestive tracts.

(7) Temperature

Warming. There is some evidence that temperature plays an important role in the spawning of *Thyone*. Several times when half of a group of animals was

kept at room temperature (20°–22° C.) while the other half remained at the temperature of the sea water (16.5°–18° C.), a number of sheddings occurred among the warmed animals, none among those kept cool. Precise tests of this were not feasible this year because of the increasing number of spent animals among those collected. The most suggestive results were obtained with 34 specimens collected on or before June 21st. At 3:35 P.M. on that date, 17 animals, group A, were placed in water at room temperature while 17 others, group B, were left in the running sea water. Five and a half hours later the water was changed on group A, some of the animals being put into fresh cold sea water, the rest into fresh warm sea water. Within a half hour shedding began in the group returned to cool water, and an hour and a half later it began in the group continued warm. In all, there were 12 out of a possible 17 sheddings. Meanwhile the animals of group B, kept cool while the others were being warmed, were placed in containers of fresh sea water and under the same lighting conditions as group A (typical overhead electric light of this laboratory). Part were put into cool water and the others into water at 20° C. There was no shedding at all during the next three hours or indeed overnight after the animals had been returned to the cold water table. Yet subsequent examinations showed that at least some of these animals could have shed, though some were already spent. The warming, if this experiment is indicative, should be of longer duration than three hours and more in the vicinity of five and a half, or more, hours.

Further support for the belief that warming induces shedding comes from the totals of the whole season's studies: No sheddings were ever observed in animals which had not been warmed for some time beforehand. Unfortunately, however, many animals which were warmed failed to shed, and others did not shed the first time they were warmed, but only after warmings on several days or after staying warm continuously for several days. Perhaps results such as these may be attributed to the waning season, or to the possibility that animals kept in confinement for many days, especially after being warmed and cooled spasmodically, may react with less predictability than fresher ones. Additional exploration of this aspect of shedding is certainly needed.

Cooling after warming. A change to cool water following a period of warming often seemed to stimulate shedding, but since most of these cases occurred at the commonest shedding time, from 8:00 to 11:00 P.M., time of day could not be ruled out as part of the stimulus. The best favoring evidence was this: A group of animals which had been in the supply house for at least a day was brought to the laboratory at 1:20 P.M. on June 22nd. They were in water of 17.8° C. which rose to 18.8° C. by 2:50 P.M. At that time all the jars of animals were placed in a cooler in which the temperature was about 19° C. but was gradually descending. At 5:45 P.M. the temperature had reached 16.5° C. and a number of animals of both sexes were shedding. Warming followed by cooling had certainly occurred but the exact time of the two periods is not known. There were also other factors. The cooler was entirely dark while housing the animals and it was vibrating vigorously. The matter of light has been discussed above and may be dismissed here. In view of the animals' normal reaction to jarring (Pearse, 1908) it seems as if the shedding may have occurred in spite of this obstacle. The time at which the shedding took place is especially interesting. It was much earlier than usual. Could it be that the treatment had overcome another possible obstacle, namely, time of day?

Continued cooling. Just as warming, or warming followed by cooling, may possibly stimulate shedding, so continued cooling appears to prevent it from taking place. If freshly collected animals are not wanted for immediate use they should be kept as cool as the water they came from, that is, no warmer than 15°–17° C., to discourage shedding. On the other hand, once an animal has started to shed, sudden cooling does not seem to stop it.

(*8*) *Other Factors*

A few attempts were made to stimulate shedding by other, more artificial, means. External mechanical stimuli such as jarring, squeezing or pricking merely resulted in quick contraction with tentacle withdrawal, wholly unfavorable to shedding. More drastic treatment, like cutting, either caused contraction as above or evisceration which makes shedding quite impossible. Injections into the body cavity can be effected without harming the animal, the amount being gauged by the size of the body. Pearse (1909), studying the physiological effects of various substances, suggested that some of his results were influenced by the fact that the animals were spawning. Conversely, it might be that some of the shedding was induced by his treatment. For instance, he found that sodium chloride, in 10% solution, resulted in "feeding movements" and otherwise caused no harm to the animals. The same reaction was obtained this year, using 0.5 to 1.5 cc. of 10% NaCl. It seemed as if the animals might be just about to shed, but no shedding ensued. This treatment should be attempted again at the height of the spawning season. Palmer (1937) found potassium chloride a shedding stimulant in sea urchins. This was not explored carefully in *Thyone* and should also be repeated earlier in the season. However, injections of KCl did not seem to be followed by the "feeding movements" that followed the NaCl injections and, moreover, some animals responded to it by eviscerating.

Summary

1. Spawning occurred in the sea-cucumber, *Thyone briareus,* during the month of June, 1948. The phenomenon appeared to wane as the month progressed. Probably early June and possibly even earlier months are the best time to find high percentages of ripe germ cells.

2. The process of shedding is described, together with various details concerning the handling of shedding animals and their gametes.

3. The following possible factors in shedding are discussed: Time of day, amount of light, presence of a shedding male to induce shedding by the female, size, quantity of sea water, freshness of animals, temperature, etc. It is shown that the first three are not essential prerequisites for shedding.

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THE PHYSIOLOGY OF EXCRETION IN MOLGULA (TUNICATA, ASCIDIACEA)

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Introduction

Twelve years ago (Das, 1936) the author wrote: "The excretory function in ascidians is attributed to a number of organs, the renal nature of some of which is still imperfectly understood. The refringent organ of the Botryllidae, the pyloric gland of the Cynthiidae, the renal vesicles of the Molgulidae and Ascidiidae, the parietal vesicles of the Cynthiidae (Pyuridae), and the neural gland of all ascidians, have been described by different workers as excretory organs." Although our knowledge of the subject has advanced considerably due to the contributions of Azema (1926, 1928, and 1929) and George (1936), so far as the fundamentals of the subject are concerned the above mentioned remarks still hold true.

The present investigation was taken up by the author during his stay in Woods Hole, in the summer of 1947, with a view to elucidating the structure, nature and relationships of the excretory organs and their products in *Molgula manhattensis* DeKay.

Lacaze-Duthiers (1874, 1892) was among the very first to locate and describe the renal organs in *Tunicata* including the renal sacs in Pyuridae (Cynthiidae). Roule (1885) described renal organs in Phallusidae, while Herdman (1888, 1899) described excretory organs in Ascidiidae, Botryllidae, and other ascidians; Dahlgrün (1901) worked on the excretory organs of Botryllidae and Ascidiidae, and compiled an account of excretory organs in *Tunicata*. Das (1936) gave an account of the excretory organs in *Herdmania*, while George (1936) described blood cells bearing excretory matter in *Tunicata*.

Küpffer (1872, 1874) was the first to demonstrate uric acid in *Tunicata*. Sulima (1914) attacked the problem of physiology of excretion in ascidians and showed that uric acid and other purine bases were the chief constituents of the excretory products. Schmidt (1924) confirmed the presence of birefringent purine derivative granules in the renal concretions of ascidians. Millot (1923) extended the investigation to other ascidians, and not only found purine bases as the chief excretory product, but discovered that in Pyuridae (Cynthiidae) the excretory granules are always made up of xanthine. Azema (1926–29) in a series of papers dealt with the mechanism of excretion in some ascidians, and demonstrated the presence of purine bases as granules inside certain blood and connective tissue cells. Finally, George (1936) has shown that certain blood cells have refractory granules of an excretory nature, and that these may circulate freely in the blood stream or be localized in the connective tissue.

From our present knowledge of the subject, therefore, *Tunciata* can be divided into two groups, based on the presence or absence of definite renal vesicles or sacs

which not only filter the nitrogenous excretory products from the blood, but also store them in a more or less insoluble form as solid renal concretions. These two groups of *Tunicata* may be called:

(a) ***the renal type***—with a definitive renal sac, and
(b) ***the arenal type***—without any such sac.

By far the greater number of tunicate species belong to the arenal type, the renal species being mainly confined to the Ascidiidae, the Molgulidae and the Pyuridae.

In the arenal tunicates, the renal function is performed by the renal cells of the connective tissue and blood, as well as the central part of the neural gland (George, 1936; Das, 1936). The localization of the excretory granules may take place in the walls of the vas deferens (*Ciona;* Roule, 1885); the mesenchyme meshwork between oesophagus and stomach on the one hand and rectum on the other (Botryllidae; Dahlgrün, 1901); the immediate neighborhood of the gut (*Ciona intestinalis;* Dahlgrün, 1901); the white pigmented patches on siphons (*Ascidia pellucida;* Azema, 1929b).

The renal tunicates, on the other hand, have a large well-marked vesicle (Molgulidae; Roule, Lacaze-Duthiers) or a number of small vesicles or sacs arranged inside the body wall or in other regions of the body (Ascidiidae and Pyuridae; Lacaze-Duthiers, Dahlgrün, and Azema). These multicellular, blind, ductless, renal sacs not only store a fluid comparable with the urine of other chordates, but strangely enough continuously deposit inside them solid concretions that accumulate throughout life (Azema, 1926).

It is remarkable that in the same family we have some species with definite renal vesicles and others which have only scattered renal cells; e.g. in the family Pyuridae, Dahlgrün (1901) records renal vesicles in *Cynthia dura* and *Microcosmus scrotus,* while Azema (1929a) found no renal vesicles in a number of Pyurid species, but renal cells were present. Similarly George (1936) could find no renal sacs in *Pyura vittata* although renal cells were again present. Das (1936) could find no renal sacs in *Herdmania pallida* (Pyuridae) but found renal cells with refractory granules not only in the visceral region, but also being discharged into the lumen of the neural gland, whence they pass out through the neural duct into the branchial cavity and thence to the outside. According to the present state of our knowledge it is highly improbable, however, that individuals of the same species may possess both the renal vesicles as well as the localized renal cells, although scattered renal cells may often be found.

Material and Methods

The renal vesicle in the Molgulidae is a large bean-shaped sac in which waste matter is gradually deposited and stored in the form of concretions throughout the life of the animal. *Molgula manhattensis* was selected because of its abundance in the Woods Hole region, and because it is comparatively easy to expose or isolate the renal sac in the living condition. The structure of the renal sac was investigated both in the living and the fixed material, while the renal concretions were examined under reflecting as well as polarizing microscopes.

The renal fluid was extracted from living renal sacs by means of a specially made micro-pipette. Care was taken to store the renal fluid under a layer of heavy

neutral mineral oil, and the method of Walker (1930 and 1937) used to determine the total molecular concentrations. The correlations between body weights and weights of renal vesicles were obtained by carefully weighing the entire live animal as well as the freshly isolated renal vesicles under standard conditions. The renal concretions were carefully separated from each vesicle, dried for a half hour at constant temperature, and weighed quickly when each batch was ready.

Structure of the Excretory Organs

The main excretory organ in *M. manhattensis* is the large, blind, and ductless renal sac situated on the right side of the body, attached to the body wall or mantle just below the pericardium. It is a thin-walled, elongated, bean-shaped sac and contains a light, yellowish-brown fluid together with the solid concretions deposited in it. It is firmly attached to the wall of the mantle and is in such close proximity to the pericardial wall anteriorly, that it is difficult to separate the renal vesicle without destroying the heart. With each wave of contraction of the heart, the wall of the pericardium is pressed against the antero-dorsal wall of the renal sac, so that the waves of contraction appear to move over this part of the sac as a series of half-ring contractions.

The renal sac lies at about two-thirds distance from the antero-dorsal border of the animal and about one-third from the postero-ventral. It lies across the mantle occupying about one third of its width at its middle (Fig. 1). In a full grown animal measuring 4 × 3 × 1.5 cm., the sac is about 16 mm. long and about 6 mm. at its greatest width. One end of the sac is bluntly rounded while the other is tapering and bent, appearing like an elongated bean in outline (Fig. 2). It is circular in cross-section in the fresh condition but tends to become flattened from side to side in fixed material (Fig. 3). In very young specimens the length of the sac is comparatively short and it appears perfectly bean-shaped; but as the animals increase in size, the sac increases more in length than in width.

The wall of the sac consists of a single layer of cells (one cell thick) which is bounded by a thin basement membrane. Each cell is almost like a cubical epithelial cell although the width is greater than the height. The nucleus lies nearer the basal end of the cell, while the cytoplasm contains one, two, or more vacuoles of different sizes (Fig. 4). The structure of each cell was best seen in the living condition, when a light methylene blue intra-vitam stain was used. The largest vacuole lying on the distal periphery of the cell always takes up the maximum stain, and contains a small mass (or two or three masses) of solid birefringent concretions that appear very much like the ones seen by George (1936) in the blood cells of *Polyandrocarpa tincta*. When kept under continuous observation, these concretions are seen to mimic "Brownian movement," increase in size, and be evacuated into the cavity of the renal sac, while a fresh vacuole soon takes the place of the one discharged. These cells are therefore primarily instrumental not only in the absorption of excretory material from the body fluid bathing the outside of the sac, but also in the isolation and final deposition of solid excretory products into the renal sac. The cavity of the sac is filled with the transparent, yellowish-brown, renal fluid.

The renal concretions lie free in the cavity of the sac. They are located in concentrated clumps or masses more at the rounded end of the sac, and spread out in a more or less continuous chain, growing towards the tapering end the the sac. The

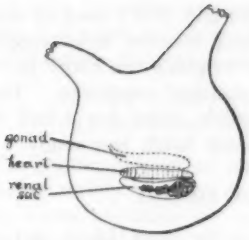

FIG. 1.

FIG. 2.

FIGURE 1. Diagrammatic sketch of right side of animal after removal of test to show position of renal vesicle (bottom) in relation to pericardium (middle) and right gonad (top).

FIGURE 2. a–f: Stages in growth of renal sac and renal concretions, (a) being the youngest and (f) full grown.

FIG. 3.

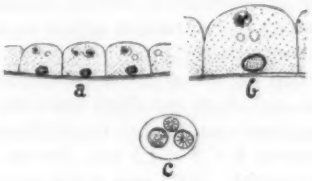

FIG. 4.

FIGURE 3. a and b: shape of fresh renal sac in T.S.; c: flattened outline after fixation.

FIGURE 4. a: the renal epithelium, × 120; b: one renal epithelial cell showing a main vacuole containing concretions, and two secondary vacuoles being formed, × 250; c: the main vacuole enlarged to show formative renal concretions, × 750.

chain can be broken up into clumps even in the living renal sac, by merely shaking it. Each clump consists of a mass of discrete rounded or oval bodies, the birefringent granules, some of which are large and others small. The smallest ones measure about 3 to 4 μ across, while some of the largest ones were 30 to 40 μ in diameter. The larger granules show definite concentric lamellae or layers of growth, the central part of the granule showing radial striations. Two, three, or more con-

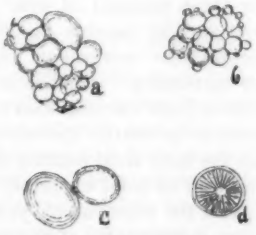

FIGURE 5. a and b: mass of renal concretions, showing attachment of later depositions on the previously deposited concretions in the renal sac; c: two concretion-bodies showing characteristic concentric layers; d: T.S. of a concretion-body showing radiating lines.

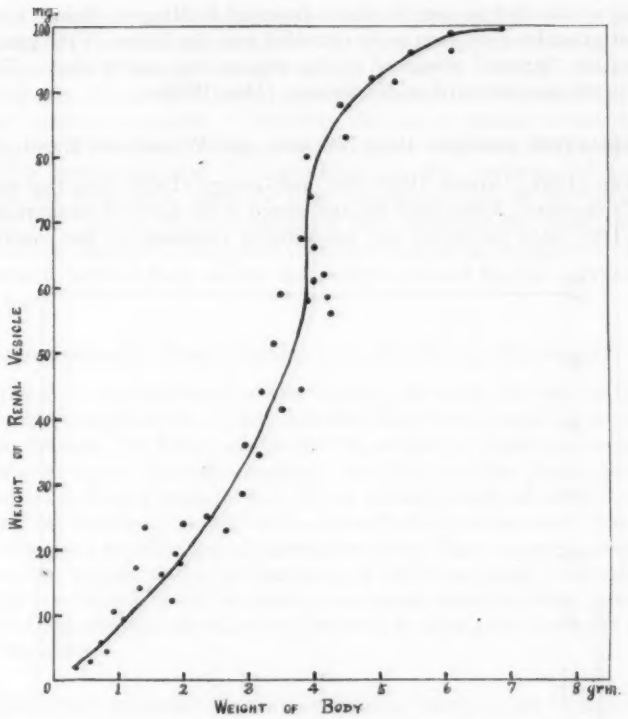

FIGURE 6. Correlation between body weight and weight of renal sac.

centric layers can be observed under the higher powers of the microscope (Fig. 5). This deposition clearly occurs from the renal fluid, without the wall of the sac taking any direct part in its formation. Smaller animals have definitely smaller excretory granules, while the largest ones show three, four, or more concentric lamellae. This appears to indicate that the rings or layers are formed by different rates of deposition of excretory matter, dependent on the differences in rates of metabolic activity at different periods in the life of the tunicate.

The neural gland opens by a neural duct into the branchial cavity, as in other tunicates. The cilia of the duct are long and lash away from the gland [1] towards

[1] Huus (1937) stated that "the ciliary pit whose function is not as yet known, may probably be an organ for the reception of sexual stimuli, and that the neural gland which is closely connected with the ciliary pit, responds to these stimuli with a hormone production which in turn calls forth spawning." Similarly, Butscher (1930), and Bacq and Florkin (1935) have shown that the neural gland in ascidians produces a hormone with similar physiological effects as pituitrin. Now a hormone is always produced by a ductless gland. The neural gland has not only a duct with cilia that beat outwards, but the entire central part of the gland consists of branching ductules joining to form the main duct. The outward beating cilia would not allow passage of fluids into the gland, although the dorsal tubercle itself may receive sexual stimuli. Pituitrin is probably produced by the peripheral part of N. gland.

the opening of the duct as seen in ducts dissected in Ringer's fluid. Cells bearing birefringent granules were seen to be extruded into the lumen of the gland and carried out by the "stream" produced by the cilia of the neural duct. This process is similar to the one observed in *Herdmania* (Das, 1936).

Correlation between Body Weight and Weight of Renal Sac

Dahlgrün (1901), Azema (1926–29), and George (1936) state that waste matter is gradually deposited in the renal sac and stored in the form of concretions throughout life. They have not given any quantitative estimates of the concretions, nor

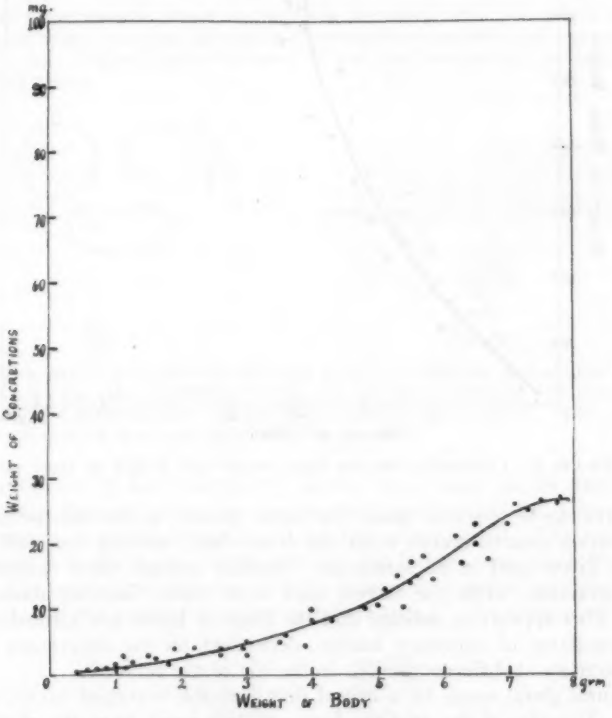

Figure 7. Correlation between body weight and weight of renal concretions.

have they established any correlations between the weight of concretions and the body weight, which alone would elucidate the rate and mode of disposal of excretory products. The present author isolated over one hundred renal sacs from animals of all possible sizes available at Woods Hole, ranging from a body weight of about half a gram to as large as seven grams. The weights of a series of individuals of graded sizes were taken and the weight of the renal sac of each was determined. The results are shown in graphical form in Figure 6. It is apparent from

the nature of the curve that the growth in weight of the renal sac is not directly proportionate to the growth in body weight. Up to a body weight of about three grams, the rate of growth of the sac is proportionate to that of the body; above this size, however, the rate of growth of the sac outstrips that of the body until a body weight of about five grams is reached. Thereafter the rate of growth of the sac becomes slower than that of the body, little growth taking place above a body weight of seven grams. This correlation may be interpreted as that between the body of the tunicate and the renal fluid, since the latter constitutes over 80 per cent by weight of the renal sac. It can thus be concluded that comparatively more renal fluid is present in the sac in half to full grown tunicates than in the younger or older stages. The correlation between body weight and weight of renal fluid is a dynamic one, as explained later.

Correlation between Body Weight and Weight of Renal Concretions

If, as found by past workers on the subject, the solid concretions in the renal sac are stored throughout life, it was surmised that there should be a straight line relationship between the body weight and the weight of renal concretions in animals of different sizes. Actually, however, the data from the present study yield a curve which is far from a straight line. From a body weight of about 0.4 gm. up to about 3 gm., the increase in weight of the concretions is rather small; but above this the weight increases rapidly and the correlation curve shows a steep ascent (Fig. 7). This means that instead of the accumulation of excretion being proportional to the body weight (i.e. instead of the increase in excretory material being proportional to the growth of the animal), the excretory material is deposited at a faster rate as the animal grows older.

Correlation between Weight of Renal Vesicle and Weight of Renal Concretions

This correlation is shown in Figure 8. It appears to be almost a straight line relationship in its first half, meaning that the rate of accumulation of concretions is directly proportional to the increase in weight of the renal sac. In other words in the first part of the life of the tunicate, the increase of renal concretions is directly proportional to the increase in renal fluid inside the sac. As the tunicate grows older, however, the rate of increase of the renal sac is overtaken by the rate of deposition of renal concretions; and we find the upper part of the correlation line curving steeply upwards (Fig. 8). This increase falls rapidly as the maximum size of the tunicate is reached; and finally the weights of both the renal sac and the concretions remain almost stationary even with an increase in body weight. The final stage, when no further accumulation of concretions is possible, may be called the *saturation stage*. The tunicate could not live much longer once this stage has been reached.

The renal sac from large living *Molgula manhattensis* was removed and the animals kept under observation for a week or more, but no regeneration of the sac or formation of secondary renal sacs was observed. This is exactly the reverse of what obtains in multivesicled animals like *Ascidia* and *Ascidiella*, where a number of renal sacs are present instead of only one as in *Molgula*. In *A. mentula* (Azema, 1926) renal sacs are formed quite early in development; but instead of all the sacs

being formed simultaneously, they are formed successively in the regions around the oesophagus, stomach, and intestine. New vesicles arise as the old ones are used up. It is apparent, therefore, that these multivesicular tunicates have a more effective excretory system than the univesicular ones.

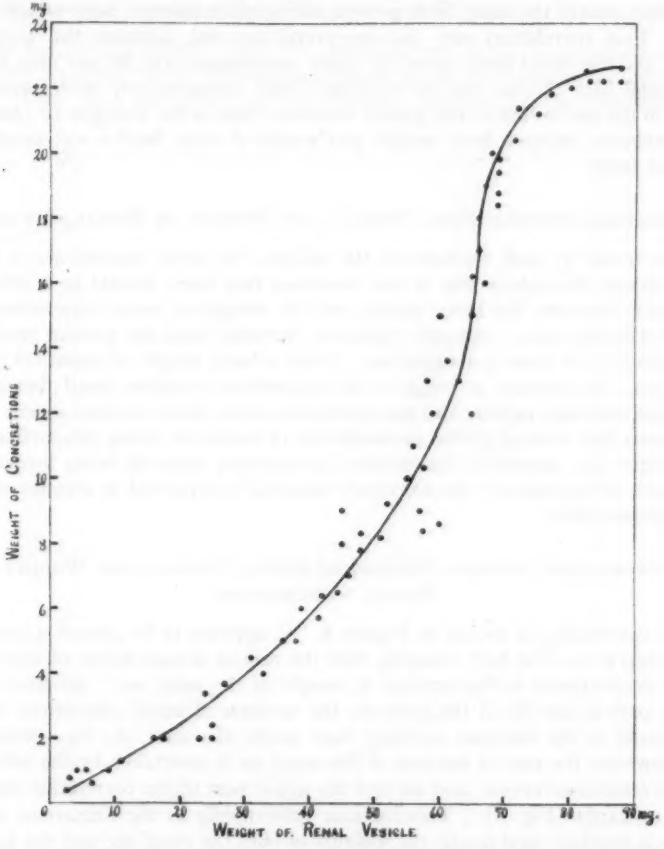

FIGURE 8. Correlation between weight of renal vesicle and weight of renal concretions.

THE RENAL FLUID

The renal fluid, when examined in fresh condition, is a clear, pale, yellowish-brown fluid which turns darker on exposure to air. The pH of this fluid ranged from 7.4 to 7.6 as determined by the Cambridge glass-electrode potentiometer. Tunicate urine is, therefore, slightly alkaline in nature. That this is caused by free ammonia was determined by the permutit method. No urea was present in any of the renal fluid samples. A full quantitative estimation of all the constituents of

the fluid could not be undertaken in the short time available at Woods Hole, but will no doubt be attempted in the near future.

Total molecular concentration and tonicity

Pure samples of the renal fluid were obtained from freshly dissected-out renal sacs by inserting a chemically clean, dry, glass micropipette into several sacs in turn. The pooled renal fluid was collected under mineral oil in order to avoid evaporation, absorption of water, or any changes due to exposure in air. The total molecular concentration of these fluids was compared with that of known NaCl solutions in "Barger tubes" (glass capillary tubes) using the method of Walker (1930 and 1937). This method involves sealing a series of micro-drops of the two fluids to be compared, each separated from its neighbours by a tiny air-gap, into chemically clean, dry, glass capillaries of very uniform internal diameter. Relative volumes of the drops are determined by measuring the lengths of the columns in the capillaries under a compound microscope, using a filar micrometer. These measurements are repeated at intervals for a period of two or three days, the capillaries being stored in a water bath between times. Consistent lengthening of a drop and shortening of its neighbours, indicates its higher total molecular concentration and vice versa. Thus by using saline solutions of known strength, the molecular concentration of the unknown fluid can be determined. Microdrops were prepared and read for:

(a) renal fluid against known saline solution
(b) body fluid against known saline solution
(c) sea water against known saline solution
(d) renal fluid against sea water
(e) body fluid against sea water

The results may be summarized in tabular form:

Specimen	Approx. corr. NaCl conc.
Pooled body fluid	2.75 gm./100 cc.
Sea water	3.10 gm./100 cc.
Pooled renal fluid	3.45 gm./100 cc.
One sample of renal fluid	Hypertonic to sea water

This comparison of the total molecular concentration of the renal fluid, the sea water, and the body fluid, has, as will be presently seen, led to very interesting results. The renal fluid was thus found to be *hypertonic* to sea water in which the animal lives, while the body fluid was *hypotonic* to sea water, i.e.,

renal fluid > sea water > body fluid.

Osmoregulation

The occurrence of hypertonic urine in *Tunicata* throws new light on osmoregulation in this group, little work on this subject having been done in the past. The body of the tunicate is closed to all exchanges between the surface and the sea water due to the covering of the test. But the current of sea water passing through the branchial siphon into the pharynx and out through the atrial siphon, brings the extensive branchial wall and vessels into close contact with it. The O_2 is absorbed

and CO_2 is thus excreted through the branchial vessels. The body fluid, as established above, is *hypotonic* to sea water; and thus it is apparent that an osmotic gradient is maintained in the tunicate between the internal and the external environment. Whether water required by the animal is taken from the sea water into its alimentary canal like the marine teleostean fishes, is not definitely established but is quite probable. The role of the hypertonic renal fluid is, however, quite definite. There is a constant flow of fluids from the blood into the renal sac, the excretory products being extracted mainly by the cells constituting the wall of the sac. The renal cells not only maintain an active osmotic gradient, but allow more or less water to pass out of the sac according to the requirements of the animal. Experimental animals were kept in grades of sea water ranging from 40 per cent to 80 per

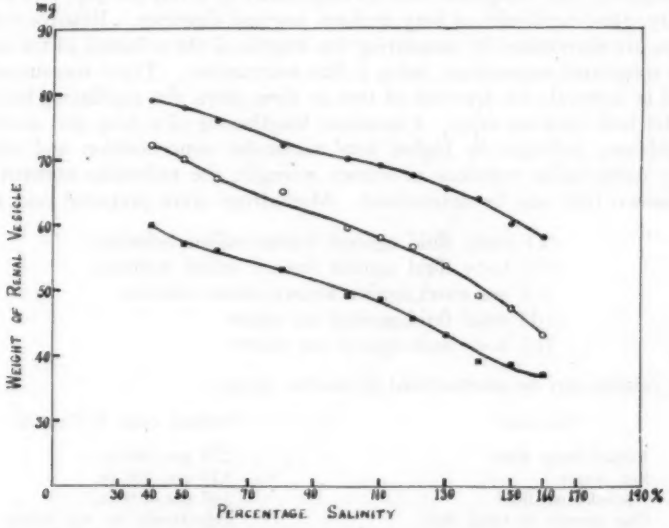

FIGURE 9. Correlation between weight of renal sac and different concentrations of sea water. Three experimental series are represented, the animals in each series weighing about the same.

cent (hypotonic), and from 110 per cent to 160 per cent (hypertonic), for a fixed period of five hours each. Each animal was weighed and a "twin" found weighing about the same. The "twins" were kept as controls in 100 per cent sea water for the same period. The renal sacs of each living experimental animal and its "twin" were rapidly dissected out and both weighed against each other. It was observed that renal vesicles from animals in diluted sea water weighed more than their controls, and that the extra weight was inversely proportional to the concentration of sea water in which the animals were kept; that is, the lower the concentration of sea water, the higher the weight of the renal sac. On the other hand, renal vesicles from animals kept in concentrated sea water weighed less than their controls; that is, the higher the concentration of sea water, the lower was the weight of the sac

(Fig. 9). It can therefore be concluded that the renal sac is an active osmoregulatory organ as well as an efficient extractor and storer of excretory material.

The Renal Concretions

As stated above, the concretions are dark brown in color, are birefringent in polarized light, and acquire a yellowish tinge by reflected light when spread out thinly on a glass slide. The concretions are insoluble in water and in dilute acids, but easily dissolved in diluted alkalies (NaOH, KOH). A qualitative analysis of the concretions was made to test for uric acid, xanthine, guanine, adenine, and creatinine. Folin's test yielded faint traces of creatinine; the murexide test and microscopic examination showed that a part of the concretions was made up of uric acid; Weidel's reaction indicated the presence of moderate amounts of xanthine; while the picrate test showed that even guanine was present. The zinc test for adenine was entirely negative, and the presence of hypoxanthine was also extremely doubtful. No allantoin could be traced in the concretions.

As shown in Figure 8, the rate of accumulation of concretions is directly proportional to increase in weight of the renal sac in the young tunicate. As it grows older, however, the rate of increase of the renal sac is overtaken by the rate of deposition of the concretions; and finally, when the saturation stage is reached, no more can be deposited.

The Mechanism of Excretion

According to the findings of Küpffer (1872, 1874), Sulima (1914), Millot (1923), Schmidt (1924), and Azema (1926–1929), the main excretory products are: uric acid in some Ascidiidae, xanthine in Pyuridae, and un-named purine derivatives in other ascidians. This picture becomes more confusing if we now add guanine (found in Molgulidae) to this list. In all animals the ultimate source of the excretory nitrogen derivatives are the α-amino-N and the nucleoproteins. A list of the main nitrogen end products of various chordates may be given here:

sharks, dogfish—urea
bony fishes (Teolosts)—ammonia
frogs, newts—urea
turtles—urea
snakes, lizards—uric acid
birds—uric acid
mammals—urea

In tunicates, however, it appears that different families have, as the main nitrogen end product of excretion, either uric acid or one of the purine derivatives.

In chordates, where we find uric acid, urea, or ammonia as the main nitrogen end product, the conversions stop at these respective steps. In the tunicate, although some of the nitrogenous matter gets converted into uric acid, a large part of it appears to stay as xanthine and guanine. We thus find in *Tunicata* a unique case of incomplete conversion, which accounts for the presence of xanthine and guanine, as well as uric acid. The author hopes to make exact quantitative estimations of these three constituents, in different tunicates, in the near future.

Summary

1. The present contribution includes a review of past work on excretion in *Tunicata.*

2. In *M. manhattensis* the single-celled wall of the renal sac absorbs the excretory products, develops the concretions, and discharges them into the cavity of the sac, where they are stored throughout life.

3. The concretions consist of granules, some of which show three or four concentric lamellae caused by further deposition of excretory matter on top of the originally secreted granule.

4. The correlation between body weight, weight of renal sac, and weight of concretions is given. It is shown that accumulation of concretions is not uniformly maintained throughout the life of the tunicate.

5. If the adult renal sac be entirely removed, no regeneration or formation of secondary sacs takes place.

6. The renal fluid is *hypertonic* to sea water, whereas the body fluid is *hypotonic.*

7. The molecular concentration of the renal fluid is 3.45 gm./100cc.; of the sea water at Woods Hole 3.10 gm./100 cc.; and of the body fluid 2.75 gm./100 cc., corresponding to NaCl concentrations.

8. The renal concretions contain xanthine, guanine, and uric acid.

Acknowledgment

I am indebted to Dr. Parpart, director of the physiology course at Woods Hole during 1947, for allowing me a table in the physiology laboratory and giving me facilities for work. My thanks are due to Dr. Kempton, professor in charge of the section on excretion and osmoregulation, for his kind advice during the course of the investigation. I should like to express my gratitude to Dr. Kellogg, my colleague in the physiology course, for confirmation of my finding, arrived at by experiments on osmoregulation, that the renal fluid is hypertonic to sea water; and also for estimation of the total molecular concentrations of renal fluid, sea water, and body fluid of *Molgula manhattensis* by the "Barger tube" method. Finally, I must thank the staff of the Woods Hole Laboratory for supplying animals when required and for the all-round kindness shown me during my stay in the summer of 1947.

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THE LIFE HISTORY AND BIOLOGY OF A MARINE HARPACTICOID COPEPOD, TISBE FURCATA (BAIRD) [1]

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Introduction

Only a few free-living marine copepods have been reared with a view to determining the details of their complete life histories. This can no doubt be attributed to the difficulty experienced in culturing most species through the entire life cycle. Considerable literature has accumulated on the morphology of developmental stages and on certain aspects of the biology, particularly of the more important planktonic calanoid species, especially *Calanus finmarchicus*. This species has also been reared through its entire life cycle in cultures (Lebour, 1916), and its biology studied under laboratory conditions more recently by Raymont and Gross (1942). The cyclopoid, *Oithonina nana*, has also been reared through the developmental stages (Murphy, 1923). But pertinent questions relative to the age at maturity, fecundity, and life span of individuals have received scant attention for other marine species. The fresh-water copepods on the other hand have been the subject of a large amount of investigation and much detail is known about their life histories and reproduction (see especially Gurney, 1931; Ewers, 1930, 1936).

Of the marine harpacticoids, *Longipedia coronata* Claus, *L. scotti* G. O. Sars, and *L. minor* T. A. Scott have been reared sufficiently to reveal specially significant aspects of their biology (A. G. Nicholls, 1935). *Tigriopus fulvus* (Fischer) has also been studied in considerable detail by Fraser (1936) and Shaw (1938). The morphology of the larval stages of a number of other marine harpacticoids has been described by various investigators, especially Chappuis (1916), Brian (1919, 1922), Gurney (1930, 1932), and Nicholls (1941). Many of the littoral species, though less conspicuous than planktonic copepods, fill an important niche as microscavengers on the bottom, and therefore warrant a close study for ecological reasons.

Tisbe furcata, also known under the generic names of *Idya* and *Idyae* (see Wilson, 1932), is a littoral cosmopolitan species. It thrives in cultures and therefore often occurs as a contaminant in laboratory cultures of other organisms. At Scripps Institution it is constantly present in the salt-water system, aquariums, etc., to which it gains entrance from the sea through the pumping system.

In 1934 it occurred in such numbers that it contributed materially to formation of flocculent detritus in the pipes. This detritus when matted together with loosened calcareous tubes of *Spirorbis* (also established in the system) caused serious clogging of water delivery jets in the aquariums.

There is some possibility that "culture forms," characterized by small differences in proportions of appendage segments and strength of setae, may develop in *Tisbe* in these cases of isolation or semi-isolation from the "wild" population. However,

[1] Contributions from Scripps Institution of Oceanography, New Series, No. 399.

Monk (1941) considers the present form to be a new variety, *johnsoni*. Controlled tests were not made of the factors involved in development of culture forms, but the temperature range (about 17–21° C.) over which the animals were reared is small compared to that used by Coker (1934) in experiments affecting the form of fresh-water *Cyclops*.

In nature *Tisbe furcata* is usually found in shallow water among algae or on the bottom where it is frequently the dominant species, but being capable of swimming, it is sometimes found also in the coastal plankton. Sars (1903–1911), who has given a full description of the adult, found it to be the most widespread of the harpacticoids in Norway wherefrom he also reports a large deep-water form of the species.

The Developmental Stages

During its life cycle *Tisbe furcata* passes through six naupliar and six copepodid stages, the last of which is the adult. The female is about 1.0 mm. and male 0.7 mm. long. The instars are separated by only one molt as in other copepods that have been studied. The female carries the eggs in a single egg-sac attached to the genital segment. The early cleavage and embryological stages have been studied by Witschi (1934).

All drawings have been made from specimens reared in the culture experiments to be described later. The assistance of Dr. Cecil R. Monk is gratefully acknowledged in connection with the 1938 cultures and with the developmental stages.

Naupliar Stages

(Plate I, Figs. 1 to 6)

In all these stages the larvae are normally benthonic, but occasionally they are taken in the plankton near shore where they have been swept off the bottom by water currents. They are colorless, subcircular in outline and possess a small red eye spot.

Nauplius Stage I (Fig. 1).—Average length 0.062 mm.; labrum circular flap; first antennae three-segmented, the distal segment with three terminal setae; the second antennae strongly built, the endopod terminating in a prehensile hook, exopod normal. The first basis of the second antennae is provided with a large but rudimentary masticatory blade or hook with a small seta at its base, and the second basis bears two strong spines. The mandibular palp possesses a splender exopod of three indistinct segments, the distal segment bearing one short and one very long seta; the endopod bears two strong short hooks and two setae. The caudal armature consists of two rather long flaccid setae.

Nauplius Stage II (Fig. 2).—Average length 0.082 mm. The obvious structural advancements over Stage I consist of increase in strength of the masticatory blade of the second antennae and the appearance of two strong setae, one each on either side of the posterior ventral side. These setae are the earliest visible fundaments of the first maxillae. This early appearance of the maxillae is characteristic of the harpacticoids. In the strange nauplius larvae of *Longipedia* the first maxillae are present already in the first stage (Nicholls, 1935). In this respect the development of harpacticoids is similar to that of many cyclopoids. In calanoids the rudiments of these appendages first appear in the third or fourth nauplius stage.

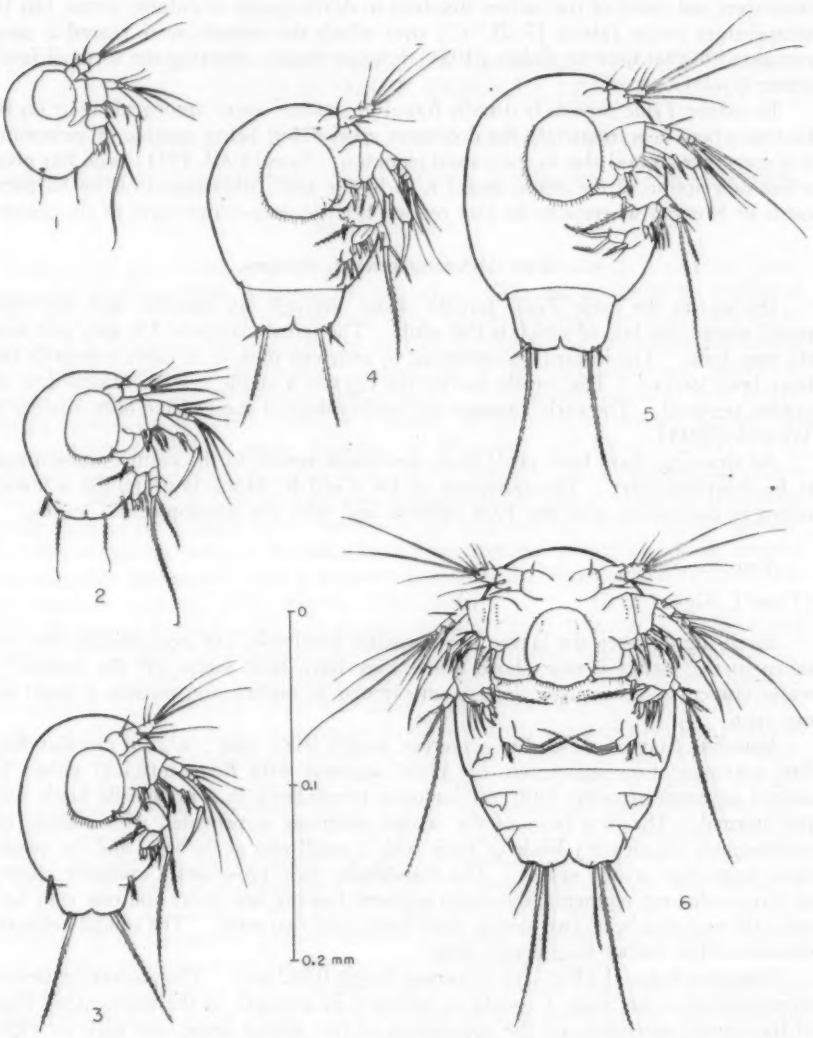

PLATE I

Development of *Tisbe furcata*

FIGURES 1–6. Nauplius stages I to VI—ventral; all drawn to same scale with aid of camera lucida.

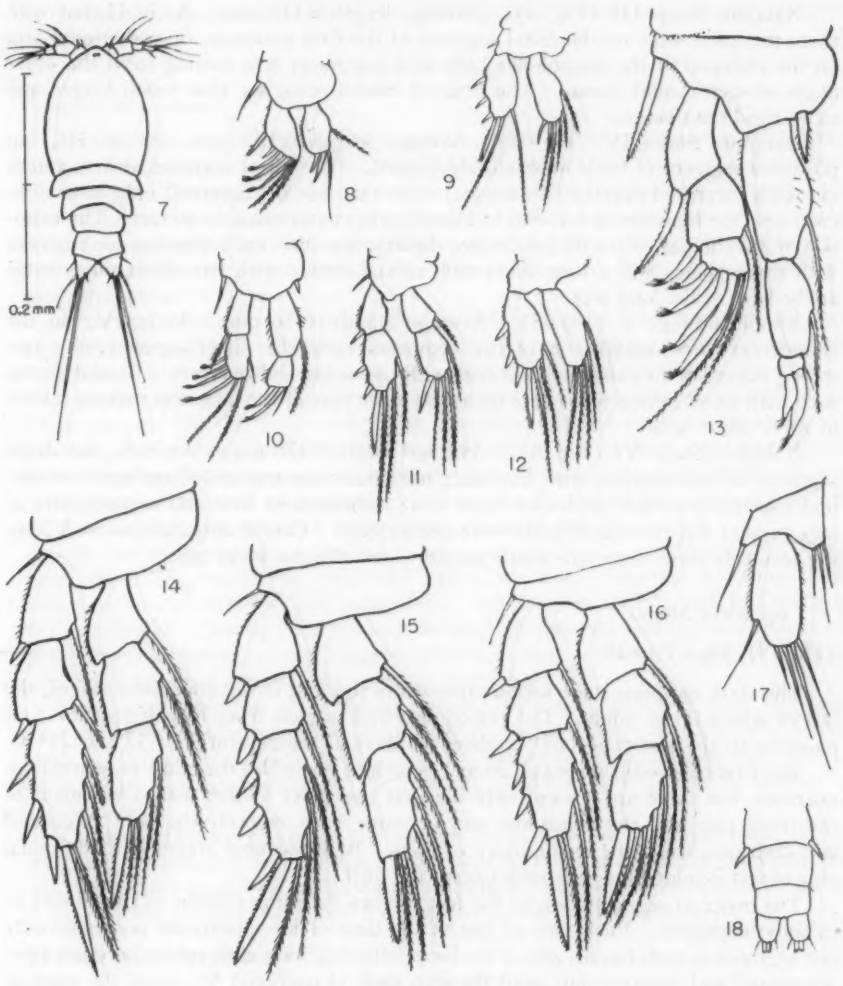

PLATE II

Development of *Tisbe furcata*

(*Camera lucida* drawings)

FIGURE 7. Copepodid stage I—dorsal.

FIGURES 8 and 9. First and second feet—Copepodid stage I.

FIGURES 10, 11, AND 12. First, second and third feet—Copepodid stage II.

FIGURES 13, 14, 15, 16, AND 17. First, second, third, fourth, and fifth feet—Copepodid stage V—female.

FIGURE 18. Posterior ventral portion of urosome of male copepodid stage IV to show sixth feet as represented by spines.

Nauplius Stage III (Fig. 3).—Average length 0.110 mm. As in II, but with three marginal setae on the distal segment of the first antennae, one additional seta on the endopod of the mandibular palp, and one short seta issuing from the outer angle of each caudal ramus. The original caudal setae are now much longer and more rigid than before.

Nauplius Stage IV (Fig. 4).—Average length 0.130 mm. As in III, but posterior segment of body more clearly defined. The distal segment of first antennae with increased number of marginal setae that can be discerned only with difficulty and are therefore not useful in identification as in calanoid larvae. The endopod of mandibular palp with four setae; the first maxillae, each showing one segment with three spines and a long seta; each caudal ramus with two short outer setae at the base of the long seta.

Nauplius Stage V (Fig. 5).—Average length 0.156 mm. As in IV, but the rudimentary first maxillae have two segments each, the distal segment with two strong curved spines and the proximal with one spine and one seta. Caudal ramus each with an additional short seta set at the inner base of the long seta making a total of three short setae.

Nauplius Stage VI (Fig. 6).—Average length 0.178 mm. As in V, but distal segment of first antenna with five marginal setae; one-segmented rudiment of second maxilla is evident with one short seta; rudiments of first and second pairs of legs present but the maxillipeds were not evident. Caudal armature as in V, but the formerly short setae are much longer, especially the inner pair.

Copepodid Stages

(Plate II, Figs. 7 to 18)

The sixth nauplius stage metamorphoses to the first of six copepodid stages, the last of which is the adult. The period required to pass from hatching of the first nauplius to the last copepodid is about 16 days at temperatures of 17° to 21° C.

The first copepodid stage is 0.20 mm. long and resembles the adult in general appearance, but there are present only the first two pairs of feet with a rudiment of the third pair and the metasome and urosome have respectively only three and two divisions, using the terminology of Sars. In subsequent stages one additional pair of feet is added for each molt except the fifth.

The order of segmentation of the feet follows the same pattern as that found in calanoid copepods. Each pair of feet at the time of its appearance possesses only one segment in each ramus, and in the next following molt each ramus becomes two-segmented and remains thus until the fifth molt (Copepodid V) when the rami of all the feet, 1 to 4, acquire three segments each (Plate II, Figs. 13–16). The new segment is derived from the proximal portion of the distal segment. This is illustrated in the first pair of feet which already in the fifth copepodid stage furnish diagnostic features of the genus. The fifth pair of feet which furnish the specific characters are not completely diagnostic until the sixth copepodid stage.

The sexes are readily separable morphologically in the fourth copepodid stage. The male in this stage is not only smaller in body size but the sixth pair of feet is already clearly developed, each foot represented by a small spine (Plate II, Fig. 18).

In the female these feet are wanting or indistinguishable from the row of fine spines on the urosome.

The Biology of Tisbe Furcata

Culture experiments

Syracuse watch glasses and low stender dishes of 50 ml. capacity were used for culture dishes in order to facilitate direct examination under the low-power microscope. The water used in the dishes was filtered to eliminate the possibility of contamination with foreign eggs or nauplii from the general sea-water supply. A fairly constant temperature was maintained by keeping the culture dishes on a table of running sea water, though the animals thrive in dishes kept at ordinary room temperatures.

A variety of marine foods was tested including ground dried mussels and scallops, dehydrated kelp, fresh seaweed and fecal pellets of *Nereis*. Each of these given individually in very small amounts was quite satisfactory as food. Shavings of fresh brown seaweed or dehydrated kelp appeared to be the best single food. Fresh seaweed has the disadvantage that the slime secreted tends to entrap the organisms. In general, however, the cultures were fed various mixtures of the above items so that some measure of selection was possible for the animals. About twice a week the dishes were cleaned by removing the debris and about one half of the water with a pipette, and a fresh supply of water and food added.

Life history series were obtained in four separate years, i.e., March-May 1935, June-July 1938, April-June 1942, and Sept.-Dec. 1947. During this time 30 or more broods and some of their progeny for two generations were studied more or less completely. During these periods no marked seasonal changes were observed in the breeding habits, or rate of reproduction, though a somewhat higher average number of eggs per egg-sac was noted in December.

Since observations on the 1942 series were the most continuous, they will form the main basis for discussing the salient features in the biology and life cycle of this species. Where data can be drawn from the earlier or later cultures, they will be made a part of the summary of time involved in the various phases or periods of the life cycle.

The lapse of time given for the various periods is the minimum time, or the average of several observed minima. For example, the first appearance of a nauplius in a culture was set as the beginning of the naupliar period and the first appearance of a copepodid marked the end of the naupliar period for any one brood.

Number of eggs produced

In order to obtain an estimate of the average number of eggs produced by each female per brood, counts were made of the eggs in the brood sacs of ten ovigerous females selected at random from the general population in March 1942. The number ranged from 29 to 82, with an average of 42.9 eggs per sac. There is obviously a very wide range in the number of eggs produced per egg-sac depending upon how long the individual has been laying eggs, and perhaps also upon the season. A check count was made in December 1947 in which the numbers ranged from 45 to 93 with an average of 72 eggs in each of 12 egg-sacs. Kunz (1935) reports 30 to 41 eggs for the species in Kiel Bay.

A group of ten ovigerous females was selected at random from the same population from which the 1942 egg counts were made and isolated for experimental culture, two females per dish. Each female was removed from the dish as soon as her eggs had all hatched. Thus each of the five culture dishes contained the nauplii from two egg-sacs. Careful account was kept of each population. As they matured and the adults mated, each mating pair was removed, recorded, and placed in another dish. In this way an accurate census was obtained.

Success of hatching and survival

From the ten broods 343 individual animals reached maturity. This indicates about 80 per cent survival, if we assume that the average egg-sac contains 43 eggs.

Incubation period

The duration of the incubation period was approximated by noting the first appearance of the egg-sac and the subsequent hatching of the nauplius larvae from the sac. The minima obtained ranged from two to four days. On the basis of the average of 21 cases the period was 2.5 days. Usually the cultures were examined every one to three days.

Naupliar period

The time required for completion of the separate stages was not studied. During the naupliar stages the animals creep about actively over the bottom or cling to detritus, etc., by means of prehensile hooks on the second antennae (Plate I, Figs. 1–6). The duration of this period is from three to eight days, with an average minimum of five days based on 22 brood histories.

Sex ratios

Of the 343 individuals reaching maturity 114 or only 33.3 per cent were females. One culture dish produced 34 females and 33 males, but in all of the rest the males were in great majority. In order to compare this unequal sex ratio in the culture dishes with that of the general population an examination was made of 44 adults from the water system. Of these only 41 per cent were females. Additional analysis involving 98 specimens reared in December 1947 also showed a ratio of one female to two males.

The numerical dominance of males is of considerable interest since usually in other species of copepods the females are the more numerous, sometimes overwhelmingly so. This inequality of the sexes is often seasonal, but no seasonality has come to light in the study of *Tisbe*.

In a study of the tide pool harpacticoid *Tigriopus fulvus*, Tsen-Hwang Shaw (1938) found 58.8 per cent females in a collection of 318 specimens.

Sexual maturity

A study of the rate at which sexual maturity was attained by 87 pairs of the 343 individuals hatched from the ten egg-sacs mentioned above, showed that two

pairs began mating (as indicated by clasping) just 14 days after they had hatched. The last four pairs mated in 25 days. The average time required to reach mating maturity after hatching was 16 days. The minimum period observed in earlier experiments at Scripps Institution was ten days. The period of clasping varied from a few hours up to one or more days, but there was no evidence that the actual transfer of the spermatophore to the female is delayed until she molts to the last or sixth copepodid stage. Clasping was rarely observed before the female had reached the sixth copepodid stage, and on only one occasion was there evidence of molting during the clasping period. This point deserves further study. Williams (1907) found that for the harpacticoids, *Harpacticus uniremis, H. gracilis,* and *Tachidus littoralis,* "every successful copulation must be prolonged until the female molts."

Length of generation

The lapse of time between the hatching of one generation and the first appearance (hatching) of the next generation resulting from the above matings varied from 17 to 24 days at temperatures of 17° to 18° C. during the spring months. The maximum period observed in one case was 31 days. In 1935, two individuals required 20 days and one individual only 16 days. MacGinitie (1937) in an incidental statement records that this species completes its life cycle in ten days at Corona del Mar, California.

The time required for *Tisbe* to develop from egg to production of eggs is comparable to that reported for various species of the freshwater cyclopoid *Cyclops.* Ewers (1936), for example, found that for 12 species under observation the time varied from 8 to 50 days.

It appears that *Tisbe furcata* may complete its life cycle in about one-half the time required for *Tigriopus fulvus* which under favorable conditions in the laboratory at University College of Hull required about two months (Fraser, 1936). However Tsen-Hwang Shaw (1938) states that for this species at Pacific Grove, California, the adult stage is reached in about one month. It is probable that the Pacific Grove species is distinct from *T. fulvus,* since Monk (1941) mentions only *T. californicus* from the coast.

Number of broods produced

In order to determine the approximate number of broods produced by a single female, four of the above 87 pairs in copula were isolated in individual dishes. Several additional males were added to each dish to afford ample opportunity for additional matings, and the males were replaced from time to time with younger mature males. (Lowndes, 1933, and Fraser, 1936, infer that the presence of males stimulates egg production.) Nauplii were removed from the dishes as soon as they appeared. Records were kept of the number of egg-sacs produced by each female under these conditions. One female produced 12 broods between May 9 and June 10. Two other females each produced nine broods in the same period and one produced five broods in 20 days before dying, apparently prematurely. The first egg-sacs produced were the largest, and the last two or three produced were about half the size of the first. The egg-sacs appeared with regularity, two to five days elapsing between hatchings with three days as the average period.

Although males were kept in the presence of the females during the whole period, only one mating was observed to take place and this preceded the entire period of egg laying. In order to test further this observation in a more crowded condition as often occurs in nature, four separate cultures were set up each containing from 8 to 20 pairs of newly matured adults in copula. A total of 54 pairs were involved. All pairs in any one dish were from the same brood and therefore of the same age. At two day intervals all adults were transferred to fresh culture dishes so that their generation could be kept separate from the younger generation constantly being produced. These adults were maintained from May 12 to June 12, and during the time following the separation of the pairs originally mated, only one instance of a second clasping was observed. This instance occurred 21 days after the original mating, and it is not known if a spermatophore was transferred. The pair concerned was isolated from the rest of the culture in order to determine if the female would continue to produce eggs for a longer time than the other females as a result of this second mating. However, no additional eggs were carried by her. This is in agreement with the earlier observations where in one case a succession of seven broods was produced by a female that had been reared in culture and isolated following one mating. She lived 70 or 71 days but produced no eggs after the 53rd day. In another test 11 ovigerous females isolated from the general population produced two to five broods, but it is not known if previous broods had been produced before isolation. Nicholls (1935) has reported a case in which a female specimen of *Longipedia scotti* had produced at least nine broods during a period of isolation from contacts with males.

The question naturally arises as to whether or not the male of *Tisbe* produces a succession of spermatophores and remains fertile after the first mating. To test this, a single male was isolated from a group of newly matured virgin males. Several virgin females were added to the dish at intervals, and records were kept of the fertility of eggs subsequently produced by each female. Out of a total of 13 virgin females brought into contact with this one male, six produced fertile eggs which hatched. The other seven females produced eggs which failed to hatch. In another test one male fertilized eight females which produced fertile eggs.

Virgin females kept in isolation have produced full egg-sacs, but in no instance was there any evidence of parthenogenesis as reported by Roy (1931) for the harpacticoid *Canthocamptus bidens*. Pine (1934) has reported parthenogenetic reproduction in *Cyclops viridis*. This, however, appears to be a misinterpretation resulting from failure to note that several egg-sacs with fertile eggs may be produced following only one mating. The females with which she worked were doubtless already fertile since they were isolated in the gravid condition from the wild.

Discussion

Rather little is known regarding the kinds of predators that feed directly upon the littoral copepods. That this type of animal food constitutes an appreciable source of nourishment for littoral animals is suggested by the fact that only a moderate population of *Tisbe* has been observed in the sea despite a relatively rapid and steady rate of replenishment as suggested by the present study. Being detritus feeders with a wide range of acceptable food, it is probable that in favorable

habitats *Tisbe* population density is controlled largely by predators. Among these predators are no doubt small fish capable of capturing small prey of this kind by pursuing and picking up the copepodid stages individually, and coelenterates having tentacles supplied with nematocysts and viscid surfaces that enable capture by chance contact. Various detritus feeders scooping up particles of detritus would consume together with this many *Tisbe*, especially the nauplii, that are prone to cling to particles upon which they are feeding.

From the present study it appears that an average of about 513 eggs is produced by each female in her lifetime. This is calculated from an average of 57 eggs per brood (using the averages of March and December) and a total of nine broods. For unknown reasons only about 80 per cent of the larvae hatched in the cultures survived to adult state. Thus, using the above figure for one female, 410 survive, of which only about 135 (approximately one third) are females. Considering a succession of generations each 25 days, a prodigious number of progeny is possible, for in about 100 days (fourth generation) over 1 billion 55 million individuals of both sexes would be produced. Ten males plus 5 females make up a mass of about 1 mm.^{3}. Hence in 100 days 70 liters of copepods would have been produced.

TABLE I

Potential rate of reproduction in Tisbe furcata and Calanus finmarchicus in cultures

	No. of broods or spawnings	No. of eggs per brood or spawning	Total no. of eggs in life-time of one ♀	Interval between generations	Survival to adult
Tisbe	7–12 average 9	29–93 average 57	513	15–31 days usually 19–24 days	80%
Calanus	1–3 (4?)	1–120 usually 15–70	Maximum observed 120	Minimum 41 days	?

These figures can of course have no significance other than to emphasize that the animal substance which *Tisbe* could supply is considerable and that a very large number of copepods must be consumed regularly by enemy predators in order to keep a balance between consumption and reproduction.

It is instructive to compare certain vital aspects of the life history of this important littoral species with similar aspects of *Calanus finmarchicus*, the most widely studied of planktonic-copepod species.

In view of the vast swarms of *Calanus* which sometimes occur in the sea, one might expect that studies of its rate of reproduction would reveal aspects suggesting possibilities of greater individual fecundity than occurs in *Tisbe*, a seemingly less abundant species. In no instance, however, is a greater rate of reproduction shown for *Calanus* on the basis of studies that have thus far been made in cultures.

Table I compiled mainly from Nicholls (1933), Clarke and Zinn (1937) and Raymont and Gross (1942) shows the great discrepancies that are brought out when comparing these two genera of widely different ecological habits.

The life span of *Calanus* is greater than that of *Tisbe*, but this appears to have no bearing on the fecundity since the number of spawnings is not shown to be affected thereby. Raymont and Gross found that under laboratory conditions *Calanus* was capable of spawning throughout the year, similar to our observations for *Tisbe*, but field observations by numerous investigators show that the rate of reproduction for *Calanus* in the sea varies greatly with season, there being a marked minimum in autumn and winter when the animals survive in copepodid Stage V. No similar field data are available for *Tisbe*.

The usually great preponderance of females over males in *Calanus* should enhance the reproductive rate provided this preponderance obtains also for the hatching eggs and provided further that there are always sufficient males in the population. That the latter is probably true is indicated by the study of Gibbons (1933). There is some evidence that in *Calanus*, as in *Tisbe*, one mating may suffice for continued spawning, since females have been observed to produce fertile eggs three or four weeks after isolation.

The greater ratio of males to females in *Tisbe* seems to have no logical explanation, since it is shown that one mating suffices for all the broods the female can produce, and each male produces a sufficient number of spermatophores to fertilize seven or eight females.

In considering this anomalous situation with respect to the relative reproductive rate of the two genera in cultures contrasted with their abundance in nature, the answer might be found in the greater ease with which *Tisbe* can be reared in culture. Hence, a truer picture might be obtained for that genus, but one that is difficult to check in the field. It is probable that in nature *Calanus* actually reaches reproductive maturity at a younger age and produces more eggs than have thus far been shown in culture experiments or deduced from field observations. An obvious alternative is that the survival rate is greater for *Calanus* (possibly because of greater opportunity for dispersal both vertically and laterally). The conclusion must then be that *Tisbe* is heavily preyed upon in its natural habitat.

Summary

1. *Tisbe furcata* is a littoral copepod that commonly invades salt-water systems connected with the sea. It is readily reared through all of its developmental stages. Being a scavenger, it thrives on various types of food, but thin slices of fresh seaweed and dehydrated kelp were especially acceptable.

2. Following the egg, there are six naupliar and six copepodid stages, the last of which is the adult. Each stage is separated by one molt.

3. The incubation period is from two to four days, usually about 2.5 days.

4. The total duration of the naupliar stage is three to eight days, usually about five days.

5. The first indication of sexual maturity as shown by clasping by the male occurred between the 10th and 25th days, usually about the 16th day.

6. The minimum time between generations (i.e., from egg to egg production) was 15 days, but usually between 19 to 24 days.

7. The span of life of individuals varied greatly. It was studied mainly in the females, some individuals of which lived for 40 to 50 days. The oldest specimer had a life span of 70 or 71 days, but no eggs were produced after the 53rd day.

8. The number of broods indicated by egg-sacs produced by isolated females varied from 7 to 12, with an average of about 9. Following the first egg sac, the subsequent ones appeared at intervals of two to five days, usually about three days.

9. The number of eggs in a brood varied from 29 to 93 with an average of 43 in one sampling and 72 in another.

10. Each female mated but once, and this mating sufficed for fertilization of all of the eggs to be produced. Males were capable of several matings.

11. About 80 per cent of the larvae hatched survived to adult state.

12. There is no evidence of parthenogenesis.

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FURTHER CHEMICAL ASPECTS OF THE SENSITIZATION AND ACTIVATION REACTIONS OF NEREIS EGGS

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The peculiar effects of 2,4,6-trinitrophenol (picric acid) in relation to the artificial activation of the eggs of *Nereis limbata* have been described in an earlier report (LeFevre, 1945). Discovery of these effects grew from a reinvestigation of a few experiments by Heilbrunn (1925) concerning the enhancing effect of acidification on the heat-activation of these eggs. Among a series of rather unrelated organic acids tested, only picric acid exerted a reliable activity of this sort, and further experimentation with this compound revealed a rather paradoxical set of properties with regard to the egg activation. Thus, at a concentration of about M/1000 in sea water, trinitrophenol prevented breakdown of the germinal vesicle, and accompanying cytoplasmic reorganization, ordinarily produced in these cells by exposure to heat (Just, 1915), excess potassium ion, or decalcifying agents (Wilbur, 1941). At the same time, exposure of the eggs to the same concentration of trinitrophenol rendered them subsequently hypersensitive to these same activating agents, so that upon removal from the acid they could be activated by exposure to these agents in doses too small to activate untreated eggs. This development of hypersensitivity was progressive with continued exposure to trinitrophenol, until after several hours the eggs were activated simply by removal to sea water, without application of any additional chemical or physical stimulant. The eggs remained in this state of hypersensitivity in the trinitrophenol for as long as three days, by which time in sea water they would long since have cytolyzed.

Various hypotheses were considered in an effort to integrate these diverse influences of trinitrophenol on the reactions of activation into a simple coherent pattern, in relation to some of the established factors relating to this process in the *Nereis* egg. The importance of the calcium ion in this connection (Heilbrunn and Wilbur, 1937; Wilbur, 1939, 1941) directed attention to the question of a calcium picrate complex formation, but there seemed to be no physicochemical basis for this; complications confronting interpretation of the effects of trinitrophenol in terms of a calcium-release theory are considered in the previous report (LeFevre, 1945). The least involved interpretation capable of explaining the behavior of the trinitrophenol seemed to be that this substance might form a reversible combination with some substance produced in the course of the cell's metabolic activities; that this substance could precipitate the breakdown of the germinal vesicle, but that it is normally removed by chemical reaction or by diffusion from the cell before activating concentrations are attained. This hypothesis would attribute the stimulating effects of the various agents to an increased rate of production of the activating metabolite, which can then take part in the critical reactions. Inhibition of this stimulation by trinitrophenol was attributed to formation of an inactive complex between the acid and the hypothetical activating substance. This interpretation accounted for the pro-

gressive development of hypersensitivity to activating agents during exposure to trinitrophenol, since removal from the acid bath to ordinary sea water, with accompanying rapid loss of picrate, would then release the accumulated activator, and would be equivalent to the application of an activating agent. The original report may be consulted for a more complete consideration of this hypothesis in the light of the earlier experiments. The present report is an extension of the above investigation in an attempt to elucidate the nature of the hypothetical reactions suggested by the original work.

Materials and Methods

Two main methods of approach have been employed: chemical disturbance of the egg-suspension medium, either during the sensitizing procedure with trinitrophenol or during application of chemical activating agents; and treatment of the eggs with substituted phenols other than picric acid, to ascertain the molecular specificity of the activities described for the latter.

The materials and general handling procedures have been previously described (LeFevre, 1945). In those experiments reported below involving treatment of egg-suspensions with gas mixtures, the appropriate quantities of carbon dioxide, unpurified commercial nitrogen, and air were drawn into a glass vessel of several liters capacity, by removal of water through a siphon; from this vessel the mixture was similarly forced in fine bubbles through 25–35 ml. of the egg-suspension in a 10–15 cm. column in an ordinary test tube. In addition to gas mixtures, specific metabolic inhibitors tested included potassium cyanide, sodium azide, sodium iodoacetate, hydroxylamine, *p*-chloromercuribenzoic acid, cupric chloride, urethane, and diethyl ether. Substituted phenols tested included 2,4,6-trinitrophenol, 2,4-dinitrophenol, *o*-nitrophenol, *p*-nitrophenol, 2,6-dichlor,4-nitrophenol, *o*-chlorophenol, and *p*-chlorophenol.

The artificial sea water medium used in some of the experiments was made up by mixing of several pure salt solutions isotonic with sea water: NaCl, 0.52 M, 500 volumes; KCl, 0.53 M, 10 volumes; $MgCl_2$, 0.37 M, 40 volumes; $MgSO_4$, 0.96 M, 15 volumes; $NaHCO_3$, 0.52 M, 2 volumes; $CaCl_2$, 0.34 M, 15 volumes. For those experiments involving upset of the normal proportions of the ingredients of sea water, corresponding changes were made in the relative volumes of the isotonic solutions mixed, so that the resultant surplus or deficit in total electrolyte content was taken up by all the other ingredients in their usual proportions.

Results

I. Experiments concerning reactions accompanying activation

a. Effects of anoxia

The dependence of the germinal vesicle breakdown response on aerobic processes was tested only in the case of activation by removal to sea water from prolonged exposures to trinitrophenol (10^{-3} M in sea water). There was no evidence of activation when the eggs were transferred to sea water through which a mixture of 95 per cent N_2, 5 per cent CO_2 had been passed for about thirty minutes, while 100 per cent activation was ordinarily obtained in undisturbed sea water or in sea water

gassed with air or 95 per cent air, 5 per cent CO_2. This inhibition of activation in a nitrogen atmosphere was reversible for several minutes; Table I shows a typical instance of this, in which the eggs, upon removal from the anoxic bath to ordinary sea water, were activated in progressively diminishing numbers as the interval in the anoxic medium was extended.

b. Effects of metabolic inhibitors

The usual chain of events following removal of eggs from trinitrophenol baths to sea water was similarly inhibited by ether, potassium cyanide, or sodium azide; these inhibitors, however, did not prevent a slight elevation of the membrane, and the very first indications of the onset of nuclear reorganization, as previously described in eggs exposed to mixtures of sea water and isotonic KCl or sodium citrate in the presence of trinitrophenol (LeFevre, 1945). This initial disturbance was "frozen" at a very early stage, so that the germinal vesicles retained their identity, and none of the cytoplasmic rearrangements consequent to vesicular breakdown occurred. If, subsequently, the inhibitor was removed, even after several hours of

TABLE I

Reversible inhibition of activation by anoxia

Sea water under atmosphere of:	Per cent activation
Air	98
95% N_2, 5% CO_2	0
Removed, exposed to air	
after 1 min.	76
3 min.	60
5 min.	54
10 min.	32

Activating procedure: eggs removed to sea water, under atmosphere indicated, after exposure for three hours to trinitrophenol, M/1000, in sea water.

such suspended activation, the usual processes continued normally; i.e., the inhibition was entirely reversible. In the case of ether, in fact, gradual evaporation of the narcotic led ultimately to a resurgence of the activation reactions, without actual removal of the eggs to fresh sea water. A typical set of experiments is presented in Table II. It may be noted that this reversibility outlasted that observed in anoxia by a wide margin, but this may have been attributable to the more rapid onset of irreversible injury to the eggs in the absence of oxygen. The critical concentrations for inhibition were those used in the experiments cited in Table II.

The same concentrations of ether, azide, or cyanide were equally effective in preventing activation in a few tests made with mixtures of sea water and isotonic KCl or sodium citrate (Table II); reversibility of this inhibition was not tested, but the high percentages of "incipient activation" observed would indicate the same state of affairs as seen with these inhibitors following the sensitization procedure. Hydroxylamine, at 10^{-2} M, also prevented activation by these agents, but the inhibition was irreversible, and although there was no immediate obvious morphological change, inhibition in this instance probably indicated nothing more than complete inactivation of the cells.

The other inhibitors tested in this same connection, rather than preventing activation, proved to be themselves activating agents. The same nuclear and cytoplasmic changes accompanying treatment with excess potassium, heat, etc., were seen when the eggs were exposed to sodium iodoacetate, at ca. $2 \cdot 10^{-2}$ M, or to urethane, at ca. $5 \cdot 10^{-3}$ M. At these concentrations, and throughout a fairly extensive range above these figures, 90–100 per cent germinal vesicle breakdown was regularly observed and was frequently followed by extensive irregular cleavage, though no swimming forms were found. Activation by these means resembled that induced by other agents in that it failed in the presence of trinitrophenol at 10^{-3} M. *p*-Chloromercuribenzoate or $CuCl_2$ (like iodoacetate, inhibitors of sulfhydryl activity) also activated the eggs, at concentrations in the neighborhod of 10^{-5} M; but this reaction was less easily reproducible than with the other stimulating "inhibitors," as the threshold concentration for activation was only slightly lower than the lytic

TABLE II

Reversible inhibition of activation by metabolic inhibitors

Activating agent	Inhibitor	Per cent activation		
		Without inhibitor	With inhibitor	Inhibitor removed after 7 hours
Sea water, after 5 hours in trinitrophenol, M/1000, in sea water	Ether, 1%	100	0, 80*	100
	KCN, 10^{-3} M	100	0, 100	100
	NaN_3, 10^{-2} M	100	0, 50	100
KCl, 20 vol. isotonic, to 80 vol. sea water	Ether, 1%	100	0, 70	
	KCN, 10^{-3} M	100	0, 75	
	NaN_3, 10^{-2} M	100	0, 100	
Sodium citrate, 25 vol. isotonic, to 75 vol. sea water	NaN_3, 10^{-2} M	94	0, 90	

* The second figure in this column is the approximate percentage of eggs in the inhibitor showing the condition of "incipient activation" (elsewhere described).

concentrations, and there was some variability in the effective concentrations for different batches of eggs. A further difference in the activation in these instances is that that induced by Cu^{++} or *p*-chloromercuribenzoate was not interfered with by trinitrophenol. Thus activation by these agents appears not to be comparable to that otherwise induced, but more analogous to the prelytic activation seen in other instances (Loeb, 1913; Lillie, 1926).

c. Effects of ionic components of sea water

The regular response of the eggs upon removal to sea water after several hours' exposure to trinitrophenol, 10^{-3} M in sea water, raised the question of what components of sea water were essential to this response. If the eggs were removed from the conditioning bath not to sea water, but to isotonic solutions of either NaCl, KCl, $CaCl_2$, or $MgCl_2$, no stimulatory reaction was ever observed. Mixtures of

various proportions of these basic ingredients were then tested. In the absence of magnesium, there was always such a distortion of the egg contours, with extreme membrane elevation and discoloring, that it was difficult to analyze other differences that might appear. It was however evident that the addition of calcium, in the absence of magnesium, greatly augmented these disruptive changes, leading to decided cellular deformation and nuclear disarrangement resembling in some respects the changes that occur in activation, and frequently to extensive cleavage, but without any change in the appearance of the cytoplasm. The deleterious effects were further exaggerated in the presence of bicarbonate buffer, with a characteristic red-brown discoloration and vesiculation of the protoplasm. In an isotonic solution containing $MgCl_2$, NaCl, and $NaHCO_3$, with the Na^+, Mg^{++}, and HCO_3^- in proportions similar to those found in sea water, the eggs remained perfectly normal in appearance. But this mixture was not capable of inducing the reactions of activation in eggs transferred from a conditioning bath with trinitrophenol. Activation required the presence of at least a reasonable trace of calcium ion; and at a given cal-

TABLE III

Action of calcium and potassium ions in initiation of activation

Concentration of potassium ion	Per cent activation with calcium ion concentration of:			
	0*	0.01 M	0.02 M	0.04 M
0*	0	0	50	66
0.005 M	0	93	92	98
0.01 M	0	100	100	100
0.02 M	3	100	100	100

* On basis of absolute freedom of reagents from Ca^{++}, K^+-contamination, and ignoring small amount carried over in sea water with eggs. Medium was made up of NaCl, 0.47 M; $MgCl_2$, 0.037 M; $NaHCO_3$, 0.0026 M; plus $CaCl_2$, KCl as indicated for each case. Eggs removed to medium indicated after exposure for 7 hours to trinitrophenol, M/1000, in sea water.

cium ion level the reaction was augmented by increased potassium ion concentration, as in the typical experiment shown in Table III. If both calcium and potassium were present at M/100, the reaction was as easily elicited as in sea water. To some extent, increased concentration of magnesium ion antagonized this stimulatory activity, so that more calcium and potassium were required to elicit the same percentage of response. The specific necessity for calcium was verified by varying its concentration in the otherwise fairly complete artificial sea water medium described in an earlier section; reduction of the calcium level to half the normal figure produced a noticeable diminution in the percentage of response, although there was still some degree of response even with only one-tenth of the normal calcium concentration.

The necessity of calcium, in initiating the stimulatory changes upon removal from exposure to trinitrophenol, was immediate, as shown in Table IV. Addition of calcium to the system only 60 seconds after the removal of the trinitrophenol did not result in a significant amount of activation. Thus in this sense the inhibition of activation by calcium-lack may be said to be irreversible. This effect is not, however, attributable to general injury to the cells because of the lack of calcium, as the

same cells may subsequently be activated by any of the usual procedures. Also, the eggs remained fertilizable for as long as eight hours in artificial sea water as nearly Ca-free as the purity of the reagents permitted. Such eggs, however, always lost their fertilizability and cytolyzed some time before those in the control medium.[1]

TABLE IV

Immediate necessity of calcium in activation following sensitization

Condition	Per cent activation
In artificial sea water	50
In Ca-free artificial sea water	0
Removed, to complete artificial sea water, after 15 sec.	51
30 sec.	36
60 sec.	10
90 sec.	0
120 sec.	0
180 sec.	0

Eggs removed to medium indicated after exposure for 24 hours to trinitrophenol, M/1000, in sea water.

In a similar manner, depletion of the calcium content of the medium prevented activation of the eggs by Wilbur's methods, addition of isotonic KCl or sodium citrate to the sea water medium. Citrate activation was especially sensitive to low calcium concentration, failing entirely if as little as ⅔ of the calcium was removed; this was particularly curious, since the citrate in itself would be expected to render unavailable most of the calcium in the medium.

II. Experiments concerning reactions accompanying sensitization

a. Effects of anoxia

Comparisons were made of the rates of development of sensitivity to sea water, during exposure to trinitrophenol, in the presence of various gas mixtures. Gassing the trinitrophenol solution (10^{-3} M in sea water) with nitrogen, or with 95 per cent nitrogen, 5 per cent carbon dioxide, led to a decidedly earlier development of sensitivity (Table V); but with more prolonged exposures, the eggs in the anoxic baths became irreversibly inactivated, and began to cytolyze, long before any evidence of damage appeared in the control aerated dishes. Increased percentage of CO_2 in mixtures with air, at least up to 50 per cent CO_2, similarly enhanced the development of sensitization, and did not appear to injure the eggs subsequently. It was frequently observed in other experiments that the packing of eggs in considerable thicknesses at the bottom of the container, during exposures to trinitrophenol, led to more rapid development of sensitivity than appeared when more dispersed, thinner layers of eggs were used. It seems likely that this effect is to be attributed

[1] On the other hand, less pronounced depletion of the calcium, down to about 1/100 the normal level, progressively delayed the onset of cytolysis; this effect may be due simply to the calcium requirements of micro-organisms responsible for the disintegration of the eggs, but Schechter (1937a, b) has described similar effects of Ca^{++}-reduction in a number of species of commonly used marine eggs.

TABLE V

Enhancement by anoxia of sensitization in trinitrophenol

Duration of sensitizing bath min.	Per cent activation following sensitization under atmosphere of: 95% N_2 5% CO_2	95% air 5% CO_2
4	0	0
16	7	1
41	100	6
59	100	63

Eggs removed to fresh sea water after indicated exposure to trinitrophenol (10^{-3} M in sea water) under atmosphere described.

to the higher CO_2 or the lower O_2 tension (or both) in the immediate environment of the cells packed in greater density.

b. Effects of metabolic inhibitors

Short of blocking subsequent activity by killing the cells (as indicated by loss of fertilizability, rapidly followed in most instances by disintegration), no reliable influence on the rate of sensitization in trinitrophenol was observed in the presence of ether, urethane, or sodium iodoacetate. (See section *d* below.) Hydroxylamine, at 10^{-2} M, or potassium cyanide, at ca. 10^{-3} M,[2] markedly enhanced the rate of sensitization, the more so as the concentration was increased; a less reliable similar activity was seen with sodium azide, in the neighborhood of $5 \cdot 10^{-3}$ M. Table VI shows a typical set of results with various concentrations of KCN. In all of these cases, the stimulatory action was superseded by lethal effects, more readily at the higher concentrations, the maximum stimulating action then passing progressively to the lower concentrations, as in the instance presented in Table VI. This cytolysis occurred following the transfer to sea water, not in the trinitrophenol baths containing the inhibitor; this is as would be expected if a great excess of the activator led to cytolysis rather than activation, as was also suggested in the older experiments.

TABLE VI

Enhancement by cyanide of sensitization in trinitrophenol

Duration of sensitizing bath min.	Per cent activation when sensitized with KCN at concentration of: $5 \cdot 10^{-3}$ M	10^{-3} M	$2 \cdot 10^{-4}$ M	0
72	100	27	39	4
127	52	68	61	2
432	0	97	75	62
1554	Cytolyzed	Cytolyzed	100	99

Eggs removed to fresh sea water after indicated exposure to trinitrophenol (10^{-3} M in sea water) with added KCN as shown.

[2] Cyanide and trinitrophenol reacted slowly in these preparations, progressively developing an amber color of unknown significance, over a period of several hours. With higher concentrations of cyanide, the rate of development of this discoloration was correspondingly higher.

c. Effects of ionic components of sea water

Following the experiments described above, in which it appeared that the calcium ion was the specifically essential component of sea water in permitting the initiation of activation, possible involvement of this ion in the reactions of sensitization was tested. Removal of calcum ion from the artificial sea water medium, containing trinitrophenol at M/1000, produced no evident change in the rate of sensitization; this absence of effect was noted with calcium contents as low as 1/60 the normal level. More complete removal was impracticable, since in such low-Ca^{++} media the trinitrophenol became rapidly toxic to the cells and led to cytolysis before the sensitization had gotten well under way. This protective effect of calcium against destruction of the eggs by the acid was evident in comparison of the normal medium with that containing as much as 1/10 the normal amount of calcium ion.

Since it was evident from experiments considered above that increased CO_2 tension hastened sensitization in trinitrophenol, it seemed expedient to test the effects of alterations in the related buffer system. But simple neutralization of the trinitrophenol (readjusting the pH from 6.7–7.0 back to 8.0 as in sea water) had no demon-

TABLE VII

Effect of bicarbonate on sensitization in trinitrophenol

Duration of sensitizing bath min.	Per cent activation following sensitization in trinitrophenol, 10^{-3} M, in artificial sea water with bicarbonate content of:			
	3 × usual	Usual (0.0018 M)	0.3 × usual	None
25	0	0	0	0
92	0	0	0	0
156	2	0	0	0
212	38	4	3	1
405	100	77	3	0
1307	100	100	100	100

strable effect on the rate of sensitization. In nearly all of the experiments with trinitrophenol, this neutralization was routinely performed. In a single experiment, the amount of bicarbonate in equilibrium with atmospheric CO_2 was varied in artificial sea water media, from zero to three times the normal amount, and eggs were exposed to neutralized trinitrophenol, 10^{-3} M, in each of these mixtures. Throughout this range, the sensitization rate was more rapid, the more bicarbonate present (Table VII). The effect of CO_2 is thus probably not to be attributed to its acidifying effects in the medium, but to its action after passing intracellularly, as in the effects described by Jacobs (1920).

d. Effects of stimulating agents

One deduction from the hypothesis of the activator-substance (LeFevre, 1945), subject to experimental test, is that the activator should accumulate more rapidly if the eggs in the trinitrophenol bath are simultaneously exposed to an agent which would bring about activation in the absence of the inhibitor. As previously re-

ported, this proved to be the case in only about ⅕ of the tests when heat was used as the activating agent, the remaining ⅘ of the results showing no significant differences in either direction.

The same procedure applied to mixtures of sea water and isotonic KCl or sodium citrate gave different results with the two agents. With addition of extra potassium to the sensitizing picrate baths, there was invariably a more rapid development of the capacity to react upon removal to sea water, similar to that shown with other agents in Tables V, VI, and VII. This was in accord with the predictions of the hypothesis; in no case, however, did such a difference appear in the presence of the citrate stimulant. Also, as noted above, the stimulants urethane and iodoacetate did not regularly enhance the development of sensitivity in trinitrophenol; each of these substances exerted this effect in a few instances, and in no case produced a slowing of the sensitization, so that their position in this regard is the same as that of the heat stimulus originally investigated.

III. Experiments concerning the effectiveness of substituted phenols other than picric acid

a. Blocking of activation

Several substituted phenols differing from trinitrophenol in the nature or position of the substituted groups on the phenol were compared, all at 10^{-3} M, the concentration at which standard experimentation with trinitrophenol was carried out.[3] 2,4-Dinitrophenol and *p*-nitrophenol were more effective than trinitrophenol in blocking activation by either KCl-sea water mixtures or sodium citrate-sea water mixtures; in one case only, *p*-nitrophenol allowed a small number of eggs to reach the stage previously designated as "incipient activation." *o*-Nitrophenol was almost as effective against citrate activation, and was superior in this respect to trinitrophenol; in spite of this fact, *o*-nitrophenol had absolutely no influence on activation by excess potassium. 2,6-Dichlor,4-nitrophenol was approximately equivalent to 2,4,6-trinitrophenol in its inhibitory action, usually allowing a moderate percentage of "incipients." *o*-Chlorophenol and *p*-chlorophenol usually prevented activation, but these effects appeared to be attributable to irreversible damage to the eggs. Prolonged exposure to the chlorophenols led to cytolysis, the initial stages of which, as noted by many investigators in artificial parthenogenesis, resemble in some respects the early stages of activation, so that it at times appeared upon cursory examination that these chlorophenols augmented the stimulating action of the chemical agents used. Phenol itself had no inhibitory effect whatsoever at this concentration.

In summary, the inhibitory effect of picric acid was duplicated by each tested phenol which had a nitro-group in the para-position; the only comparable action

[3] If neutralized, trinitrophenol at concentrations as high as 10^{-2} M were tolerated for many hours, and were very effective in blocking and sensitizing, but the concentration of 10^{-3} M gave the most prolonged effects without cell damage. Increasing the concentration past 10^{-3} M did not increase the rate of development of sensitivity to sea water in eggs exposed to the trinitrophenol; this independence of the rate of sensitization from the trinitrophenol concentration is in harmony with the hypothesis advanced in the discussion concerning the mode of action of the trinitrophenol.

seen in the absence of this group was that of *o*-nitrophenol with respect to citrate activation.

b. Sensitization to sea water

At 10^{-3} M, *o*-nitrophenol, 2,4-dinitrophenol, *o*-chlorophenol, or *p*-chlorophenol had no sensitizing effect on the eggs in exposures up to 24 hours or until the onset of cytolysis. Cytolysis occurred within a few hours in *p*-chlorophenol, after about 20 hours in *o*-chlorophenol or dinitrophenol, but no sooner in *o*-nitrophenol than in sea water. *p*-Nitrophenol showed some sensitizing activity, as indicated in

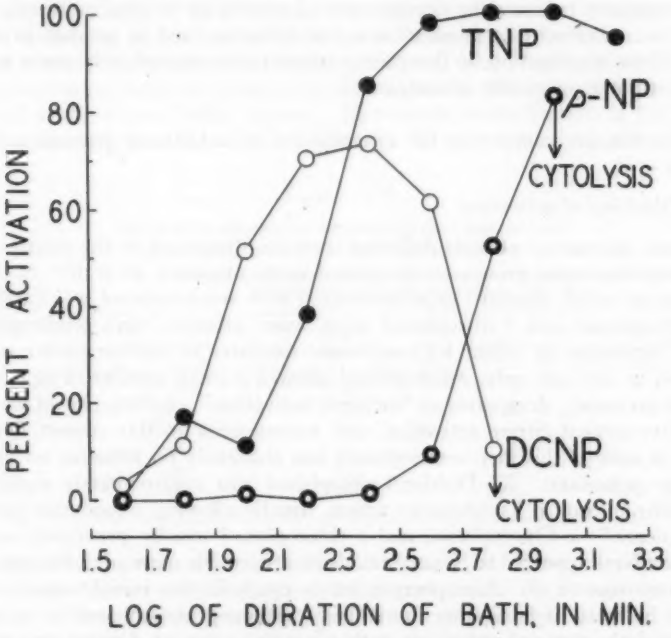

FIGURE 1. Comparison of sensitization rates in the three effective substituted phenols found.

Eggs removed to sea water following bath in either 2,4,6-trinitrophenol (TNP), *p*-nitrophenol (*p*-NP), or 2,6-dichlor,4-nitrophenol (DCNP); all at 10^{-3} M, in sea water.

Each point is the average of all experiments (usually three or four) performed in the logarithmic time interval marked at the base-line. Each individual experiment compares the action of the three phenols on the same batch of eggs.

Figure 1, but only after about 10 hours, and this effect disappeared rather rapidly, so that cytolysis began to be evident at about 20 hours. The dichloronitrophenol sensitized the eggs to sea water as rapidly as, or perhaps a little more rapidly than, trinitrophenol, but, like *p*-nitrophenol, led to cytolysis within about 20 hours (Fig. 1).

Thus, it seemed apparent that again the $-NO_2$ group in the para-position on the phenol was the primary essential, the groups in the ortho-positions exerting only modifying influences on the sensitizing reactions. However, unlike the situation observed in the study of inhibition of activation, there was no sensitizing activity evident with 2,4-dinitrophenol, which contains the apparently critical group. This is especially odd in view of the fact that either the addition of another $-NO_2$ in the 6-position, or removal of the $-NO_2$ from the 2-position, produces a highly active substance (many swimming "larvae" were obtained from the use of trinitrophenol or *p*-nitrophenol). Also, substitution of $-Cl$ for both of the $-NO_2$ groups in the ortho-positions did not interfere wth the sensitization activity.

However, of all these, only 2,4,6-trinitrophenol (picric acid) was effective in the lengthy preservation of the eggs against death and cytolysis; the others were all, in fact, somewhat toxic, leading to cytolysis within the first day of exposure. This may mean that the two nitro-groups in the ortho-positions serve to detoxify the molecule, perhaps preventing other reactions not basically related to those involved in the reversible inhibition and sensitization under study.

Discussion

None of the newly acquired data is antagonistic to the hypothesis developed from the results previously reported, involving the concept of the metabolite activator-principle. However, no crucial experimental test of this hypothesis has as yet been conceived; the present data concern the general nature of the reactions accompanying chemical activation and sensitization of the eggs, insofar as this is revealed by aberrations in these reactions with changes in the chemical environment.

Thus it is apparent that some phase of the reactions accompanying the process of germinal vesicle breakdown involves an oxidation employing molecular oxygen, probably through a cytochrome system, since this process was reversibly prevented by anoxia, cyanide, or azide. However, as noted by Barron (1932), in the activation of *Nereis* eggs by actual fertilization by sperm in very complete anoxia, this inhibition affects not the initiation of activation, but the later nuclear changes and consequent development. Diethyl ether also prevented these reactions, but there is less specificity in its action, so that interpretation of this inhibition is more indefinite.

With the exception of ether, the same inhibitors which prevented activation also hastened the onset of sensitization of the eggs exposed to trinitrophenol, so that the cells were activated, upon removal from the acid to plain sea water, following a shorter stay than required in the absence of the inhibitors. This fact is readily incorporated into the general hypothesis, since inhibition of the activating reactions would be expected to lead to more rapid accumulation of the activator-trinitrophenol complex, by eliminating one of the routes by which the free activator might be otherwise removed. Interpretation might also be sought on the basis of the acidifying effects of these inhibitors intracellularly, with consequent release of Ca^{++} from combination with cellular proteins. The fact that the inhibitors never appeared to induce sensitization in themselves, together with the temporal characteristics of the sensitization, would necessitate a rather unwieldy complex of assumptions about the behavior of the Ca^{++} and trinitrophenol, in application of these interpretations (LeFevre, 1945).

The observed results would however be expected if there were any alternative pathway by which the activator could be removed, either by chemical reaction or by diffusion from the cell; or simply if the reaction in which the activator is released were reversible and governed by mass action. In the latter case, accumulation of the activator in the presence of these metabolic poisons would be self-limited, whereas large amounts could be accumulated in inactive form with trinitrophenol, and this process would be accelerated by addition of the inhibitors; this would account for all the relevant experimental results with a minimum of independent hypotheses.

As noted above, the metabolic inhibitors which prevented activation exert their influence not on the immediate activating disturbance (visible at the egg surface) but on the immediately subsequent cellular reorganization. On the other hand, inhibition of the same over-all process ("activation") by removal of calcium ions from the medium acted at the very earliest stages in the chain of events. The absence of calcium ion at the critical instant of potential initiation of activation was thus an irreversible disturbance, and the cell did not react upon replacement of the missing element unless a second stimulus was applied. Heilbrunn (1925), Heilbrunn and Wilbur (1937), and Wilbur (1939, 1941) concluded from experiments along various lines that the reaction in question is dependent on rearrangement of the intracellular calcium with respect to the protoplasmic colloids, with accompanying changes in viscosity. The modifying influences of potassium and magnesium ions, as reported above, are in keeping with the general pattern of these cations in affecting colloidal reactions with calcium ion, as described by Heilbrunn in numerous cellular reactions.

Other aspects of the effects of metabolic poisons on the reactions of activation are not so readily interpreted; it is particularly odd, though not entirely without parallel, that germinal vesicle breakdown is initiated by exposure to iodoacetate or urethane (or, in a different manner, by application of Cu^{++} or *p*-chloromercuribenzoate), which substances are generally recognized as inhibitors or narcotics. The most evident interpretation of these results in the light of the related observations is that these inhibitory agents may prevent some alternative reactions of the activator substance or its precursors, so that the activator concentration is increased by the presence of the inhibitors. Obviously no specific characterization of the hypothetical reactions involved can be made, except that it seems likely that some enzyme concerned contains active sulfhydryl groups.

Some special mention should be made of the fact that there is not complete agreement in the results obtained with the various procedures employed. Some of these discrepancies are easily dismissed as quantitative differences in effects of the activators and inhibitors on the critical reaction rates. However, the distinct inhibition of citrate activation by *o*-nitrophenol is entirely out of line with all other relevant data; citrate in stimulatory concentrations also invariably failed to hasten sensitization of the eggs in trinitrophenol.

Beyond the considerations outlined, the present data do not permit identification of the hypothetical substances or their reactions; perhaps some clue is afforded in the apparent specificity of the *p*-nitrophenol grouping in the reversible formation of an inactive complex with the activator. The author is not prepared to interpret this finding; innovation in experimental approach is probably necessary

before a more coherent pattern will emerge from the diverse observations reported in this paper and in the earlier report. The interpretations offered seem the least involved and most comprehensive of the facts available at this time.

Summary

1. Inhibition of activation of *Nereis* eggs by trinitrophenol, with concurrent sensitization of the eggs to subsequent stimulation, appears to depend on the nitro-group in the para-position on the phenol.

2. The rate of the sensitization process is enhanced by anoxia, CO_2, inhibitors of the cytochrome system, or increased potassium ion concentration, but is insensitive to several other inhibitors, narcotics, stimulating agents, and to calcium ion deprivation.

3. The immediate initiation of activation by various chemical procedures requires the presence of the calcium ion, is assisted by the potassium ion, and slightly depressed by increasing magnesium ion concentration, but is not affected by anoxia or by various metabolic poisons.

4. Subsequent nuclear and cytoplasmic reorganization, ensuing some minutes after the initial disturbance, is reversibly inhibited by anoxia, inhibitors of the cytochrome system, or diethyl ether.

5. Urethane and iodoacetate activate the eggs; this activation is inhibited by trinitrophenol. Cupric ion and *p*-chloromercuribenzoate also activate the eggs, but only at nearly lytic concentrations, and the activation is not affected by trinitrophenol.

6. These data are partly interpreted in relation to the hypothesis of an activator metabolite produced within the egg.

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THE ACTION OF CHOLINE AND RELATED COMPOUNDS ON THE HEART OF VENUS MERCENARIA [1]

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Since the demonstration by Prosser (1940) of the unusual sensitivity to acetylcholine (Ach) of the isolated heart of the bivalve molusc, *Venus mercenaria,* we have extensively employed this preparation for the bio-assay of Ach in tissue extracts (e.g. Welsh, 1943; Welsh and Hyde, 1944a and b; Prajmovsky and Welsh, 1948). In certain respects it is superior to the classical Ach assay preparations such as the dorsal muscle of the leech, rectus abdominis of the frog, isolated frog heart, and blood pressure of cat. For example, it is more sensitive to Ach, with complete inhibition occurring at about 50 times the threshold inhibitory concentration; it is relatively unaffected by changes in pH, inorganic ions, and tissue constituents other than Ach; it recovers quickly, thereby allowing more rapid estimation than the above-mentioned preparations.

While employing the *Venus* heart for bio-assay, its responses to a variety of drugs, organic compounds, and inorganic ions have been studied and, in particular, to a series of choline esters and analogs—this in the hope of obtaining evidence toward a better understanding of the fundamental mode of action of Ach. An organ with a high specificity for choline esters, exhibiting a response which is easily quantified, and which has so little self-contained cholinesterase that blocking of this enzyme is not necessary when working at great dilutions of the unstable esters, provides a suitable object for studying certain aspects of the mechanism by which Ach acts on cells.

The present paper has two purposes: (1) to indicate the methods of preparing and employing the *Venus* heart for the bio-assay of Ach, and (2) to compare the effects of other choline esters and related compounds which differ in greater or less degree from the Ach molecule.

Methods of Preparing and Using the Hearts for Ach Estimation

An earlier paper by Wait (1943) covers some of the necessary procedures for preparing the isolated *Venus* heart for Ach determinations. For convenience, however, the steps which we employ from the securing of appropriate test animals to the quantitative estimation of Ach in tissue extracts will be outlined.

Venus mercenaria (the hard shell clam or quahog), being an important commercial shellfish along the Atlantic Coast, are usually available where shellfish are sold. They remain edible for some weeks after digging or dredging, if maintained under refrigeration, but after one to two weeks the hearts of such animals tend to beat with an irregular rhythm; it is important, therefore, to obtain experimental

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material from a source where the previous history is known [2] and to use this material within one to two weeks after removal from the sea. *Venus* with a shell length of 8 to 12 cm. have been found of most convenient size. In the laboratory they may be stored dry at 5°–10° C., or preferably kept in shallow tanks of aerated sea water, at 15°–18° C.

The heart is exposed by breaking and removing the dorsal portion of the shells (umbos and hinge) and then cutting away the mantle and pericardium dorsal to the heart. The heart consists of a single, median ventricle with laterally disposed, thin-walled atria (auricles). Anterior and posterior blood vessels leave the heart in close association with the intestine which passes through the heart. Threads for attaching to a support in the bath and to the writing lever may be passed under the atria and tied close to the ventricle in order to include some of the thicker-walled, ventricular muscle. Cutting the atria distal to the threads, and cutting the blood vessels and intestine, isolates the ventricle which may then be placed in an appropriate heart bath. Only the outer surface of the heart is directly exposed to materials introduced into the bath, but cannulation of the heart and introduction of Ach into the ventricle does not increase its sensitivity.

The bath figured by Wait (1943) is satisfactory unless temperature control is desired (e.g. when working in a room above 20° C., or when maximum sensitivity is required); then a bath with a water jacket through which water of an appropriate temperature (15°–18° C.) is circulated may be employed, or the heart bath may be placed in a larger temperature-controlled vessel. It is necessary that provision be made for changing the fluids of the heart bath without draining the bath and subjecting the heart to undue mechanical disturbance. A bath holding 10 ml. when filled has been found appropriate.

An analysis of the inorganic salts of the blood of *Venus mercenaria* by Cole (1940) showed only small differences in comparison with sea water. It is not surprising, therefore, that sea water is an adequate perfusion fluid for the isolated heart, allowing a regular beat to be maintained for 2–3 days. Where natural sea water is not available, an artificial perfusion fluid may be used and several have been tried, with differing ratios of the common ions, without noting any appreciable effects on the heart until radical departures from the normal concentrations of the common ions are made. A fluid found satisfactory has the following composition: 30 gm. NaCl; 0.9 gm. KCl; 1.1 gm. $CaCl_2$; 3.5 gm. $MgSO_4 \cdot 3H_2O$ in one liter of water with a phosphate or bicarbonate buffer (pH 7–7.5). Changes of pH between 6 and 8.5 have little or no effect on the amplitude or frequency of beat, or on the response of the heart to acetylcholine for periods of time up to several hours.

Oxygen may be supplied by air or a mixture of 95 per cent O_2–5 per cent CO_2 passed through the bath. The bubbles should be small to avoid mechanical disturbance to the heart. The gas mixture or air may be admitted to the bath through the hooked support for the lower attachment of the heart if this is made from glass tubing drawn out to a fine tip. The heart lever should be counterweighted to give a pull of 200–300 mg. A kymograph speed of about 2 cm. per minute is desirable. Substances to be tested may conveniently be added at the bottom of the bath by

[2] E.g. Supply Department, Marine Biological Laboratory, Woods Hole, Mass., or a wholesale dealer in shellfish.

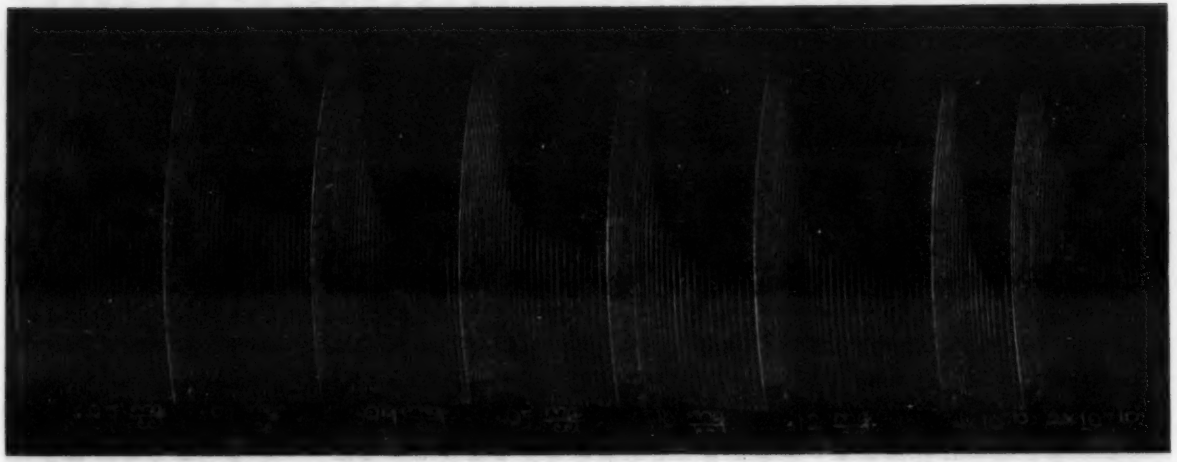

FIGURE 1. Sample record of bio-assay using the isolated *Venus* heart to estimate the amounts of extractable acetylcholine in two isolated ventral nerve cords (A and B) of nymphs of the cockroach, *Blaberus*. Total wet weights of tissue available: cord A = 17.3 mg.; cord B = 15.4 mg. The sensitivity of this heart to Ach and the approximate activities of the extracts had been determined in an earlier portion of the record. At the first arrow an amount of extract of cord A was added to the bath to give a final dilution of substance per cc. yielded by an original wet weight of tissue of 0.07 mg. The extract remained in the bath approximately one minute; the drum was stopped and the fluid of the bath changed several times and the heart allowed 3–5 min. to recover its original amplitude. After recording a few normal beats the extract of cord B was added at the second arrow. Following two attempts to determine amounts of extracts of cords A and B required to produce equivalent decreases in amplitude of beat, two known concentrations of Ach were applied (4×10^{-10} and 2×10^{-10} gm./cc.). From the results it was possible to calculate that cord A contained an Ach-like material equivalent to 4.0 μg. Ach/gm. wet weight while cord B yielded a value of 3.3 μg. Ach/gm. On a second heart the values obtained were A = 5.0 μg. Ach/gm.; B = 4.2 μg. Ach/gm.

means of a long hypodermic needle bent at a right angle; the volumes added should be small (1 ml. or less).

In estimating the Ach content of a tissue extract, a dilution should be found that gives between 20 and 80 per cent decrease in amplitude of beat at the end of one or two minutes. This should be matched, preferably twice, with known concentrations of Ach, after which appropriate calculations will give the Ach equivalent per gram of tissue. A sample record is shown in Figure 1. If it is suspected that substances in the tissue extract other than Ach are affecting the heart, it may be desirable to treat a portion of the extract by the addition of NaOH and warming in order to destroy the Ach present, and after neutralizing with HCl, to use this to make up the last dilution of Ach prior to adding to the bath.

Treatment of a heart with an anti-cholinesterase (physostigmine, neostigmine or di-isopropyl fluorophosphate) may potentiate the action of Ach two to five times. This small degree of potentiation is undoubtedly due to the low level of cholinesterase activity in these hearts (Smith and Glick, 1939; Jullien et al., 1938). Because the untreated heart is so sensitive to Ach (threshold for inhibition is usually between 10^{-10} and 10^{-11} gm. per ml.), and because recovery after Ach is slowed by treatment with an anti-cholinesterase, it is normally undesirable to employ this means for increasing sensitivity.

Occasionally hearts are encountered which fail to beat or which beat with a low amplitude. Although adrenalin and tyramine have been found to be excitants in relatively high concentrations (5×10^{-5} to 10^{-4} M) their effects are quickly abolished by washing. On the other hand ergotoxine, ergotamine, and ergonovine have been found to have a remarkably persistent excitatory action. For example, one part per million of ergotoxine ethanesulfonate will frequently cause renewal of heart beat, or an increase in amplitude of two to three times, in a heart with an abnormally low amplitude. Treatment for a few minutes with one of these ergot alkaloids produces a change in the physiology of the heart which persists for many hours in spite of repeated washings, while the response to Ach is affected but slightly. In this connection it should be noted that the *Venus* heart is composed of smooth muscle and its pharmacology is not unlike that of certain types of vertebrate smooth muscle.

There is some seasonal variation in the sensitivity of *Venus* hearts to Ach, with maximum sensitivity in the late winter and spring months (cf. Prosser, 1940; Wait, 1943), but the change is probably not as great as indicated by Prosser. More important to note is that in late summer there is a tendency toward irregularity in beat. This is often so pronounced that accurate estimates of small differences in Ach levels cannot readily be made in August and early September in the region of Massachusetts.

The Action of Certain Choline Derivatives and Related Compounds on the Venus Heart

The greatest gap in our knowledge concerning Ach is the precise manner in which this physiologically active substance affects the excitability of cells. More detailed studies of the mechanism of action of Ach are needed, and it would appear to matter little what type of Ach-sensitive tissue or organ is used in these studies. For many reasons the isolated heart of the quahog appears to be peculiarly suitable

for such a study, and in this section of the present paper it will be shown that no compound related to Ach has yet been found having as great an inhibitory action on this organ as does Ach. It will also be shown that the methyl grouping around the onium element is, in many respects, the most significant portion of the Ach molecule.

The typical effects of Ach on the *Venus* heart will first be described, and then the relative activities of a number of common choline derivatives and certain related compounds will be discussed.

When Ach is added to a bath containing a beating *Venus* heart, which has received no previous drug treatment, to give a concentration in the vicinity of 10^{-11} to 10^{-10} M, a small increase in amplitude is sometimes observed. A similar stimulating action of low concentrations of Ach on vertebrate hearts has been observed by McDowall (1946) and others. At concentrations of Ach in the vicinity of 10^{-9} to 10^{-8} M a negative inotropic effect is seen; and with increasing amounts of Ach the amplitude of beat decreases until the heart stops in diastole at a concentration of Ach about 50 times that which gives a just measurable decrease in amplitude. Thus the range of concentrations from the threshold of inhibition to complete inhibition is relatively narrow. The log-concentration-response curve is sigmoid, with the portion between 20 and 80 per cent inhibition approximating a straight line. The inhibitory action of Ach on the *Venus* heart is more prominent and consistent than the excitatory action of lower concentrations, but it seems probable, as pointed out elsewhere (Welsh, 1948), that Ach first excites and then in higher concentrations inhibits or paralyzes this organ as it may do to all tissues or organs which respond to Ach.

In Table I a summary is given of the relative inhibitory activities of a number of compounds related to choline or Ach. The data shown in this table were obtained by finding a molar concentration of Ach that would produce between 20 and 80 per cent decrease in amplitude of beat of a given heart, and then the molar quantity of a related compound that would produce a degree of inhibition exactly matching that produced by the Ach. Each value shown for a given compound was obtained on a different heart. The average values may be taken as a fairly precise indication of the relative inhibitory effectiveness of these several compounds on the *Venus* heart. Since cholinesterase activity in this organ is extremely low, anti-cholinesterases were not employed, but the complications which may arise when stable choline esters are compared with unstable in the presence of active cholinesterases are believed to be minimal.

In commenting on certain of the more interesting facts given in Table I attention may first be called to choline. Choline affects the isolated *Venus* heart in a manner very much like that of Ach, except that it is far less active. When first applied in low concentrations, the amplitude of beat may increase (Fig. 2, Curve 1). Greater variation in the response of different hearts to choline was observed than in the case of any other compound. This is illustrated by Figure 2, where concentration response curves for three different hearts are shown. The wide range of values obtained when choline was compared with Ach may be accounted for by individual variation in the response to choline, for it is obvious that if a match of molar concentrations of Ach and choline producing 25 per cent inhibition were made on the heart represented by curve 3, the relative value for choline might be 1000; while a match of 25 per cent inhibition made on the heart represented by curve 2 would yield a value showing Ach to be perhaps 50,000 times as active as choline.

TABLE I

Relative molar quantities required to produce a decrease in amplitude of beat equivalent to that produced by a molar quantity of acetylcholine chloride equal to 1

Choline derivative	Structural formula	Molecular weight	Values obtained	Averages
Carbamylcholine chloride	$(CH_3)_3NCH_2CH_2OCNH_2$ (Cl; O)	182.5	20 20 25 40 40 50 80 100 100 150 250	80
n-Propionylcholine chloride	$(CH_3)_3NCH_2CH_2OCCH_2CH_3$ (Cl; O)	195	100 100 100 120	105
Ethoxycholine bromide	$(CH_3)_3N(Br)—CH_2CH_2OCH_2CH_3$	212	100 100 100 130 200 200 200 250	160
Butyrylcholine chloride	$(CH_3)_3N(Cl)—CH_2CH_2OC(=O)(CH_2)_2CH_3$	209	200 500 800 1,000	625
Chloracetylcholine chloride	$(CH_3)_3NCH_2CH_2OCCH_2Cl$ (Cl; O)	215.9	500 750 1,000 1,600	960
Acetyl β methyl choline chloride	$(CH_3)_3N(Cl)—CH(CH_3)CHOCCH_3$ (O)	195	400 1,000 1,000 2,000	1,100
Benzoylcholine chloride	$(CH_3)_3NCH_2CH_2OC$—C_6H_5 (Cl; O)	243	10,000 10,000 12,000 13,000 20,000	15,000
Choline chloride	$(CH_3)_3NCH_2CH_2OH$ (Cl)	139.5	400 1,000 1,000 1,000 1,000 2,000 4,000 5,000 7,000 15,000 40,000 100,000	14,000
Betaine ethyl ester chloride	$(CH_3)_3NCH_2COCH_2CH_3$ (Cl; O)	181.5	1,000 1,000	1,000
Betaine hydrochloride	$[(CH_3)_3NCH_2CO(=O)]\cdot HCL$	117	3,300 5,000 10,000 20,000 25,000 25,000	15,000
Triethylcholine chloride	$(C_2H_5)_3NCH_2CH_2OH$ (Cl)	272	10^{-4} M—no inhibition	
Triethylacetyl choline iodide	$(C_2H_5)_3NCH_2CH_2OCCH_3$ (I; O)	315	10^{-3} M—no inhibition	

However, the average value showing that 14,000 times as many molecules of choline are required than of Ach to produce a given effect, is probably a close approximation to the relative activities of these two compounds on the *Venus* heart. Thus the acetic acid ester of choline is far more active than is the parent compound. This is an observation that has been made by many workers on many tissues and organs.

The several esters of choline which were tested and the one ether (ethoxycholine) were all far more active than choline, with the exception of benzoylcholine which has approximately the same level of activity. The presence of the ring structure at the non-polar end of the molecule obviously affects the activity greatly. It is of interest to note that the substitution of chlorine for a hydrogen atom of the terminal methyl group in Ach to yield chloracetylcholine reduces the activity approximately one thousand fold.

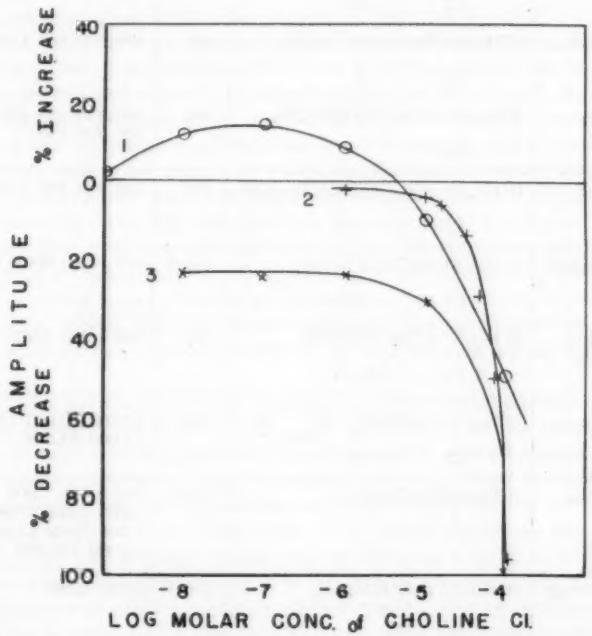

FIGURE 2. Showing the extreme variation in response of three different isolated *Venus* hearts to choline. Heart number 1 responded by an increase in amplitude to low concentrations of choline while hearts 2 and 3 showed only a decrease in amplitude, but their sensitivities differed markedly.

Betaine, a naturally occurring compound closely related to choline, is approximately equivalent to choline in its ability to depress the beat of the *Venus* heart, while the ethyl ester of betaine was found to be considerably more active than betaine.

A clear indication of the importance of the methyl groups attached to the nitrogen was seen when triethylcholine and triethylacetylcholine were tested. In the highest concentrations employed (10^{-2} M), neither of these produced the slightest degree of inhibition of heart beat. We have observed a similar striking difference in the actions of the tetramethyl ammonium ion, which decreases the amplitude of beat of the *Venus* heart, and tetraethyl-, tetra-n-propyl-, and triethyl-n-octyl ammonium

ions, all three having an excitatory action only. These results obtained with the quaternary ammonium ions will be reported more extensively in a separate paper.

Importance of the methyl groups attached to the onium element directs attention to this portion of the Ach molecule. It is apparent from the present study and from similar earlier studies that different choline esters differ in their degree of pharmacological activity. This is also true of the *Venus* heart, but they all produce a characteristic decrease in amplitude. The substitution of ethyl for methyl groups in choline and Ach yields molecules which are completely lacking in inhibitory activity when applied to the isolated *Venus* heart. It has been indicated elsewhere (Welsh, 1948) that this specificity of the $(CH_3)_3N$ group, the rapidity of action of and recovery from Ach, and its activity in small amounts, suggest that Ach acts at the surface of cells as a "trigger" to set off a reaction or chain of reactions in the manner of an unstable coenzyme. Thus, the condition of the cell membrane is altered and the cell excited and then depressed depending on the concentration and time of action of the Ach. In further testing of this hypothesis, it is believed that the isolated *Venus* heart will continue to provide an ideal experimental object.

Summary

1. A method of preparing and employing the isolated heart of the quahog, *Venus mercenaria,* for the bio-assay of acetylcholine (Ach) is described.

2. The activities of choline and certain choline esters; of betaine and its ethyl ester; and of triethylcholine and triethyl-acetylcholine on the isolated *Venus* heart are compared. In further understanding the fundamental mode of action of Ach, the most significant observation was that the substitution of ethyl groups for methyl on the nitrogen of choline and Ach resulted in a complete loss of activity determined by observation on the amplitude of heart beat.

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INCIDENCE AND ORIGIN OF ANDROGENETIC MALES IN X-RAYED HABROBRACON EGGS[1]

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Introduction

The terms androgenesis and merogony are sometimes used interchangeably. The former is defined by Wilson (1925) as "the activation of the egg by the sperm followed by development without the participation of the egg nucleus;" the latter as the "development of an egg fragment devoid of a nucleus fertilized by a normal sperm." This distinction is kept in this paper.

The study was undertaken with two objects in view: first, to determine whether low incidence of androgenetic males or high embryonic mortality is responsible for the low ratio of their occurrence as adults; and second, to work out the cytological mechanism underlying androgenesis in *Habrobracon*.

The literature dealing with androgenesis is considerable. Much of it is summarized in Wilson (1925), Sharpe (1934) and Darlington (1937). Three papers have been selected for discussion here. Packard (1918) exposed unfertilized *Chaetopterus* eggs in first meiotic metaphase to radium, and fertilized them with untreated sperm. When exposure was relatively long (35 to 50 minutes) the egg nucleus remained attached to the second polar body, the sperm nucleus divided, and development was androgenetic. Hasimoto (1934) identified some silkworm (*Bombyx*) males as androgenetic. These had developed from eggs which had been exposed to high temperature at time of oviposition when the eggs were undergoing the maturation divisions. These males were diploid, and by means of appropriate genetic combinations, he was able to demonstrate that they arose from the "union of two sperm nuclei in the egg cytoplasm without fertilization with the egg nucleus." Polyspermy is the rule in *Bombyx*. The third paper (Astaurow, 1937) describes the production of androgenetic males, likewise in *Bombyx*. They were produced along with the expected classes either by thermo-activation (40° C. for one hour) after fertilization, or by irradiation of the egg followed by fertilization with untreated sperm, or by both. At X-ray doses lethal to the egg nucleus, androgenetic males developed only after thermo-activation of the fertilized eggs. Heat treatment is used in this form to break diapause.

Material and Methods

For X-ray treatments a dual-tube self-rectifying outfit with a simultaneous cross-firing technique was used. The secondary voltage was 182 kv. and the tube cur-

[1] This investigation was completed with the aid of a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service. The author is also grateful to the University of Pennsylvania and to the Marine Biological Laboratory, Woods Hole, Massachusetts, for use of laboratory facilities, and to Mr. L. R. Hyde for administering the X-ray treatments. The drawings were made by Mrs. Jean Wilson.

rent on each tube was 25 ma. The heavy glass of the tube walls and 5 mm. of bakelite of the tube shields gave the filtering value of 0.2 mm. copper shield. The output intensity was 7210 r per minute, distance 9.5 cm. All breeding was carried on at 30° C.

Well-fed wild type females of the parasitic wasp *Habrobracon juglandis* were X-rayed and mated to untreated males which differed from wild type by one or more recessive traits. Eggs laid by these females during the first six hours after treatment had been X-rayed in late metaphase of the first meiotic division (metaphase I); the majority of those laid after this time had been treated in first meiotic prophase (prophase I) (Whiting 1938). Lethal dose [2] for the former is about 2400 r; for the latter, about 54,000 r (Whiting, 1941).

From all control crosses of the type used in this study, only diploid biparental females and haploid gynogenetic males are produced. Therefore, when wild type females are mated to males with traits recessive to wild type, daughters are wild type, heterozygous for the recessive traits, while sons have maternal wild type genes only. If this kind of cross is made after the females have been X-rayed, there appear occasionally males which show all the recessive paternal traits (Whiting, 1946a). These males are normal in appearance, fully fertile and transmit paternal traits only. They are, therefore, androgenetic. Their fertility is proof that they are haploid since diploid males which may arise from certain crosses in *Habrobracon* are always sterile or nearly so.

Repeated tests have shown that androgenetic males arise from eggs treated in metaphase I, and of 381 such eggs observed, six only, 1.57 per cent, developed into these exceptional males (dose 14,420 r–28,840 r). Although lethal dose for the nuclei of these eggs is 2400 r, androgenetic males have developed in eggs X-rayed with dose as high as 54,000 r (Whiting 1946b).

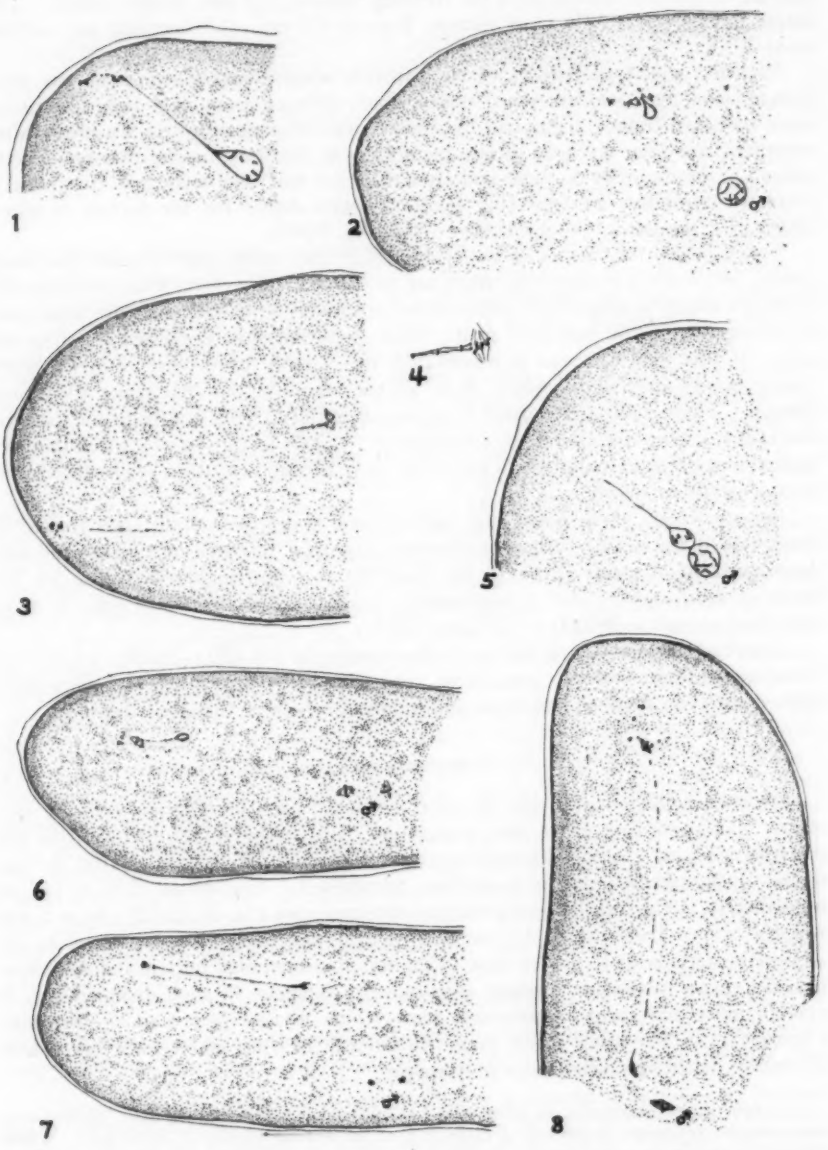
Eggs laid during the first six hours after treatment (14,420 r–36,050 r) were collected at one hour intervals, punctured, and fixed in Kahle's fixative. They were stained with the Feulgen technique and mounted whole in Canada balsam.

Observations

Speicher (1936) found that the most advanced eggs in *Habrobracon* egg sacs are in "early anaphase of the first maturation" (the author prefers to call this late metaphase I), and described normal oogenesis following oviposition. After the egg is laid, the maturation spindle passes into telophase I. The second division follows immediately. The four haploid groups of chromosomes (1a, 1b, 2a, 2b) lie in a row roughly perpendicular to the egg surface. During anaphase II, polar nuclei 1a and 2a remain stationary, 1b moves close to 2a, and 2b (functional nucleus) sinks deeper into the egg, a membrane forming as it moves. Nucleus 1a soon disintegrates, 1b and 2a unite and form a metaphase plate which divides and then disintegrates. Cleavage is of the usual insect type, with nuclei moving about until blastoderm formation, when cell membranes first appear.

[2] Apparent inconsistencies in lethal doses in successive papers dealing with *Habrobracon* eggs are due to changes in method of calibration at the Marine Biological Laboratory. Conditions of treatment have not varied. In this paper all doses have been corrected for the latest measurements.

PLATE I



After treatment with X-rays in prophase I, chromatin fragments, bridges, or both may occur in division I, or in division II, or in both divisions (Whiting, 1945a). Bridges occur but rarely in division II and when present appear to be single. After treatment in metaphase I, chromatin fragments (never bridges) may be seen between chromosome groups in telophase I and chromatin bridges appear in division II. These bridges may occur between nuclei 1a and 2a, or 1b and 2b, or between both pairs, never between 2a and 1b. They are made up of several chromatin threads and are present in both regions when dose is high. The attached egg pronucleus can move a long distance without breaking the bridges, and under these conditions it is pulled out into a "tear-drop" (Whiting, 1945b). Controls, of course, do not show fragments or bridges.

These bridges may retard the female pronucleus, but that they do not often stop it completely at relatively low doses is demonstrated by the fact that after lethal dose (2400 r) all but 2.4 per cent of unfertilized eggs develop well beyond first cleavage before death, and that these 2.4 per cent advance to first cleavage before dying (Whiting, 1945a).

These facts suggested to the author that the chromatin bridges formed during the second meiotic division of eggs X-rayed in metaphase I might sometimes retard the attached egg pronucleus to such a degree that the untreated sperm pronucleus would cleave before the egg pronucleus could reach it.

There were 702 eggs prepared and studied. Of these, 58.55 per cent were useless, either because they had been fixed at stages previous to syngamy or cleavage, or were not clearly stained; 41.45 per cent were of significance. They included all eggs undergoing syngamy as well as those in cleavage, where either chromosome number or presence or absence of chromosome aberrations demonstrated which pronuclei had taken part in cleavage. Of the 291 eggs fulfilling these requirements, three (1.03 per cent) only were found in which androgenetic development had begun (Plate 1, Figs. 6, 7 and 8). Three eggs not included in the 291 suggested incipient androgenesis. In each the male pronucleus was preparing for first cleavage while the female pronucleus was greatly retarded (Plate 1, Fig. 2). If these are accepted as androgenetic, a maximum of six among 294, or 2.04 per cent, is obtained. This does not differ significantly from 1.57 per cent of adult survivors, and demonstrates

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PLATE I

All illustrations were drawn with aid of a camera lucida from whole mounts of eggs which had been X-rayed in first meiotic metaphase. Fertilization was accomplished by untreated sperm. Anterior end and lateral view of each egg is shown. Cytoplasm is somewhat conventionalized. The lenses employed were a Spencer 2 mm. n. a. 1.3 apochromatic oil immersion objective and a × 5 or × 10 compensating ocular.

1. Attached pronucleus of unfertilized egg. Dose 28,840 r. × 650.
2. Attached and greatly retarded female pronucleus with normal male pronucleus. Egg X-rayed with 28,840 r. × 650.
3. First cleavage spindle of unfertilized egg. Chromatin bridges still attached to some cleavage chromosomes. Dose 14,420 r. × 370.
4. Cleavage spindle of Figure 3 in detail. × 650.
5. Syngamy. Egg X-rayed with 36,050 r. × 650.
6. Second cleavage of androgenetic development. Egg pronucleus greatly retarded. Egg X-rayed with 36,050 r. × 370.
7. Second cleavage of androgenetic development. Egg X-rayed with 28,840 r. × 370.
8. First cleavage of androgenetic development. Egg X-rayed with 14,420 r. × 370.

that there is little or no death of androgenetic embryos in spite of their development in cytoplasm irradiated with doses from six to fifteen times that lethal for the egg nucleus.

A study of Plate I will illustrate some points of cytology. It should be recalled here that *Habrobracon* chromosomes are extremely small. This will explain why they are not always represented exactly as to form and number ($n = 10$). In Plate I, Figure 1, is shown a typical "tear-drop" pronucleus. Polar nuclei are degenerating and suggest by their condition that the egg pronucleus is considerably retarded. Chromatin at the outer end of, as well as within, the egg pronucleus, is attached to bridges. This egg was not fertilized. Figures 3 and 4 show what can happen to such a tear-drop as that just described. The spindle is that of the first cleavage, and bridges with chromatin thickenings can be seen, still attached to chromosomes on the spindle. This is the only egg studied in which the chromatin connections could not be followed continuously from polar nuclei to egg pronucleus. What happens to a tear-drop pronucleus in the majority of cases when an X-rayed egg is fertilized after treatment, is shown in Figure 5. It unites with the normal male pronucleus and, in doing so, ultimately kills the embryo because of upset in chromosome balance due to chromatin loss.

Figure 2 strongly suggests an incipient androgenetic male. The egg pronucleus is so retarded and the male pronucleus so advanced that subsequent syngamy seems highly improbable.

Figures 6, 7, and 8 represent the only eggs found in which androgenetic development had begun. The isolation of the egg pronucleus from cleavage figures, with no evidence of any chromatin connection or remains between them and the normal appearance of the cleavage chromosomes, are to be noted. Figure 8 is especially convincing.

These three eggs have one thing in common which is rare in control eggs and not the rule in X-rayed ones: cleavage is taking place more posteriorly than one would expect. This suggests that some cytoplasmic factor, perhaps greater fluidity of the cytoplasm, may alter action of the sperm pronucleus so that it has moved "beyond the reach" of the impeded egg pronucleus.

Discussion

It is definitely established that androgenetic development (haploid) occurs in *Chaetopterus* and in *Habrobracon* after irradiation in metaphase I. Concerning *Bombyx*, Kawaguchi (1928) states, "Die Kerne in den Ovarialeiern der Schmetterlinge nach ihrem Ausschlüpfen aus der Puppe stehen fast immer im Stadium der Metaphase der ersten Reifteilung." This indicates that in *Bombyx* also treatment was given in metaphase I, since adult females were irradiated and then mated to untreated males.

That there is some special cytological response of tetrads to irradiation which causes chromatin bridges to be formed in division II, is apparent in both *Chaetopterus* and *Habrobracon*. The author (1945b) has discussed this in some detail but has been prevented from checking the theory completely by the small size of *Habrobracon* chromosomes. *Chaetopterus* chromosomes are relatively large and distinctive in character and should be analyzed in detail from this viewpoint. Packard describes chromatin bridges (dicentrics) in cleavage in eggs irradiated with doses low enough

to permit the egg pronucleus to function in syngamy, a fact consistent with the conditions found in *Habrobracon*.

No cytological study of *Bombyx* eggs after treatment has been made. Hasimoto's conclusion, derived from genetic data, that androgenetic males (diploid) arise from the union of two sperm pronuclei, is not inconsistent with the suggestion that, in this form also, chromatin bridges retard the egg pronucleus.

That some viscosity change may be involved in *Habrobracon* androgenesis as well as chromatin attachment of the egg pronucleus to the polar nucleus, has been mentioned above. Hasimoto and Astaurow obtained androgenetic males in *Bombyx* by thermo-activation alone during meiotic divisions and after fertilization, and this may be evidence for viscosity change and movement of sperm pronuclei from their usual position. However, in *Chaetopterus* the sperm chromosomes remain in their normal position, close to those of the egg, yet syngamy does not occur.

A comparison of other factors in the two insect genera shows further similarities. In both, the percentage of eggs developing into androgenetic adults is low—1.57 per cent in *Habrobracon;* never higher than 0.273 per cent in *Bombyx* (Astaurow, 1937). In the former, 54,000 r is the highest dose at which androgenetic males were obtained; in the latter, 27,000 r was the highest dose tested, and they were produced after this treatment. In the *Chaetopterus* study, treatments were measured in minutes of exposure to radium bromide. After 50 minutes' exposure, about 70 per cent of treated eggs underwent haploid cleavage. At longer treatments the percentage dropped, until prolonged exposure stopped cleavage altogether.

In *Chaetopterus,* incidence of androgenesis but not adult survival; in *Bombyx,* adult survival but not incidence; and in *Habrobracon,* both incidence and adult survival, have been determined.

Summary

1. 1.57 per cent of *Habrobracon* eggs X-rayed in first meiotic metaphase (14,420 r–28,840 r) and laid by treated females mated to untreated males develop into androgenetic males. These will develop after any dose up to 54,000 r although lethal dose for the egg chromosomes in this stage is 2400 r. Cytological study of 294 such eggs (14,420 r–36,050 r) shows that three were undergoing androgenetic cleavage while three others were possibly preparing for it. A maximum of six, or 2.04 per cent, does not differ significantly from androgenetic survivors, and it must be concluded that androgenetic embryos at doses used are as viable as embryos developing in untreated eggs.

2. Chromatin bridges which appear in meiotic division II after treatment in first meiotic metaphase retard and distort the egg pronucleus, occasionally to such a degree that the sperm pronucleus cleaves and develops into a normal fertile haploid male with paternal traits only. The almost complete absence of these bridges after treatment in first meiotic prophase will explain the failure of androgenetic males to develop in these eggs.

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ERRATUM

PAPERS PRESENTED AT GENERAL SCIENTIFIC MEETINGS, MARINE BIOLOGICAL LABORATORY, SUMMER OF 1948

OCTOBER, 1948, ISSUE. PAGES 264–265

The relation of the plasma membrane, vitelline membrane and jelly in the egg of Nereis limbata. DONALD P. COSTELLO.

This article contains abstracts of two papers presented by Dr. Costello. The title "Spiral Cleavage" should be inserted following line 6, page 265.

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